Valorization of Traditional Portuguese Apples and Cherries

Biochemical characterization and development of functional ingredients

Ana Teresa de Carvalho Negrão Serra

Dissertation presented to obtain a Ph.D degree in Engineering and Technology Sciences, Biotechnology at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa

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Front cover: *Bravo de Esmolfe* apples, *Saco* cherries, high pressure extraction cell and manometer, XAD16® resins and apple and cherry lyophilized extracts

Back cover: Apple and cherry liquid and lyophilized extracts

by Ana Teresa Serra

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FOREWORD

This thesis aims to contribute for the valorization of traditional Portuguese fruits. Apples and cherries that are only cultivated in specific regions of the country were investigated for their bioactive effects and as sources of functional ingredients with promising application in food, pharmaceutical and nutraceutical industries.

À memória dos meus avós Clara e Camilo

Aos meus pais

À Guida

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ABSTRACT

The consumption of fruits and vegetables is widely recognized to be beneficial to human health and this is mainly attributed to their high content in functional constituents, such as vitamins, carotenoids and polyphenols.

In Portugal, the annual production of apples and cherries is about 300,000 and 17,000 ton, respectively. Between all varieties produced, there are some traditional fruits, which are only cultivated in specific regions of the country. In general, these traditional varieties are very appreciated by consumers due to their unique sensorial properties. However, they are less produced and thus more expensive than exotic cultivars. Within this context, this thesis focused on the valorization of these traditional Portuguese fruits in order to increase their productivity and competition in the market.

In a first approach, traditional and exotic apples and cherries were evaluated for their bioactivity. Different analytical chemical and biochemical methodologies were developed and applied to characterize fruits in terms of phenolic content, antioxidant activity and antiproliferative effect against human colon and gastric cancer cells.

In the case of apples, 26 varieties were screened for their polyphenolic content and antioxidant potential. From the results obtained four traditional varieties (*Bravo de Esmolfe*, *Malápio Fino*, *Malápio da Serra* and *Pêro Pipo*) and five exotic fruits (*Fuji*, *Gala Galaxy*, *Golden*, *Reineta Parda* and *Starking*) were selected and fully characterized. The traditional varieties *Bravo de Esmolfe* and *Malápio Fino* showed to be rich sources of functional ingredients with powerful antioxidant activity and relevant antiproliferative effect. The exotic *Reineta Parda* was the best apple in inhibiting human gastric and colon cancer cells proliferation.

Bioactivity and chemical characterization results were correlated and demonstrated that catechin, epicatechin and procyanidin B1 are the major

contributors of the antioxidant activity of apples whereas procyanidins (B1 and B2), phloridzin and epicatechin play an important role in inhibiting human cancer cell proliferation.

As the presence of these compounds could be regulated by environmental and climatic factors, results obtained from two consecutive crop years were also evaluated. Biological activity differences were observed however less significant than those occurred between different varieties.

In cherries, two traditional cultivars (*Saco* and *Morangão*) were studied and compared with seven exotic varieties (*Summit*, *Maring*, *Van*, *Early Van Compact*, *Lapin*, *Ulster* and *Garnet*). *Saco* cherry together with *Ulster* and *Lapin* showed the highest content in phenolic compounds, the best antioxidant activity and the most effective response in inhibiting the growth of human colon and gastric cancer cells. When correlating bioactive response and phenolic composition, anthocyanins were identified as the major contributors of biological activities. Additionally, the presence of hydroxycinnamic acids, flavan-3-ols and flavonols demonstrated to be determinant for the protection against oxidative stress.

Within the aim of this thesis clean separation processes were applied to isolate biofunctional ingredients from rejected traditional fruits (fruits that do not fit all the requisitions to be sold as fresh edible products). *Saco* cherry culls and *Malápio Fino* apples were used as raw material due to their recognized rich content in bioactive compounds.

In order to develop biofunctional ingredients that can potentially show anticancer effect, natural extracts containing perillyl alcohol, were isolated from *Saco* cherries using high pressure extraction. The methodology employed comprised a first step with supercritical CO_2 followed by a second step where different mixtures of CO_2 and ethanol were tested. The effect of a supercritical CO_2 treatment as well as the influence of the ethanol

concentration in solvent mixture composition was studied concerning extract yield, phenolic content, antioxidant activity and antiproliferative effect in human colon cancer cells. The product derived from CO_2:ethanol (90:10 v/v) extraction exhibited the highest antiproliferative activity, being 150 times more effective than fresh fruit. In addition, cherry extract showed to be a promising natural agent to be included in cancer chemotherapy as it induced cell cycle arrest in a different check point than a common drug. In an effort to further increase the antiproliferative potential of cherry extract, different processing strategies were explored. Indeed, altering solvent purity the antiproliferative effect was significantly improved by 16 fold. Furthermore, by adding a conventional extraction step (using methanol or ethanol:water mixture) prior to multi-step high process it was possible to obtain cherry extracts with even higher antiproliferative activity (up to 2 fold).

Finally, the recovery of powerful antioxidant concentrates from *Malápio Fino* apple and *Saco* cherry was also explored. Using a macroporous resin it was possible to obtain polyphenols-rich extracts (40-50% w/w) with enhanced antioxidant activity accessed by cell-free and cell-based assays. Transport studies in Caco2 cell model were performed demonstrating that apple and cherry extracts can cross the intestinal barrier to further deliver their protective effects.

RESUMO

Ao consumo de frutas e vegetais é reconhecido um efeito benéfico para a saúde que desde há muito se relaciona com a presença de ingredientes funcionais, nomeadamente vitaminas, carotenóides e polifenóis.

Em Portugal são produzidas anualmente cerca de 300,000 toneladas de maçãs e 17,000 toneladas de cerejas onde se incluem algumas variedades tradicionais cultivadas apenas em determinadas regiões do país. De um modo geral, estes frutos são bastante apreciados devido às suas características organolépticas únicas, mas, sendo produzidos em menor escala tornam-se mais dispendiosos do que as variedades exóticas.

Deste modo, o principal objectivo do trabalho desenvolvido nesta tese consistiu na tentativa de valorização das variedades tradicionais de maçã e de cereja com vista a promover a sua produção e aumentar a sua competitividade no mercado.

Numa primeira fase, foram avaliadas as propriedades bioactivas das várias variedades tradicionais e exóticas de maçãs e cerejas produzidas em Portugal. Diferentes métodos químicos e bioquímicos foram desenvolvidos e utilizados de forma a caracterizar os frutos relativamente ao teor em compostos fenólicos, capacidade antioxidante e efeito antiproliferativo em células humanas do cancro do cólon (HT29) e do estômago (MKN45).

Em relação às maçãs, o estudo incidiu inicialmente em 26 variedades, das quais se seleccionaram nove, sendo esta escolha tida por base a quantidade de polifenóis totais e o potencial antioxidante que apresentaram. As nove maçãs incluíram quatro variedades tradicionais (*Bravo de Esmolfe*, *Malápio Fino*, *Malápio da Serra* e *Pêro Pipo*) e cinco variedades exóticas (*Fuji*, *Gala Galaxy*, *Golden*, *Reineta Parda* e *Starking*) e foram objecto de uma caracterização mais exaustiva. As variedades tradicionais *Bravo de Esmolfe* e *Malápio Fino* demonstraram ser maçãs ricas em ingredientes funcionais com

elevado potencial antioxidante e um efeito antiproliferativo significativo. A maçã *Reineta Parda* foi a variedade que apresentou um maior efeito na inibição do crescimento de células humanas do cancro do cólon e do estômago.

Foi possível correlacionar a bioactividade determinada para as diferentes variedades de maçã com a composição em polifenóis e verificou-se que a catequina, a epicatequina e a procianidina B1 foram os principais compostos responsáveis pela actividade antioxidante. A epicatequina e a procianianidina B1, juntamente com a procianidina B2 e floridzina, mostraram, também, contribuir para o efeito antiproliferativo das maçãs.

Neste trabalho avaliou-se ainda o efeito do ano de colheita uma vez que a concentração destes compostos nos frutos pode variar com as condições ambientais e climáticas. Apesar das diferenças encontradas entre os resultados obtidos em dois anos consecutivos, estas foram menos significativas do que as verificadas entre as diferentes variedades de maçãs.

No caso das cerejas, foram estudadas duas variedades tradicionais (*Saco* e *Morangão*) e sete variedades exóticas (*Summit*, *Maring*, *Van*, *Early Van Compact*, *Lapin*, *Ulster* and *Garnet*). A cereja *Saco*, juntamente com as variedades *Ulster* and *Lapin*, demonstrou elevada concentração em compostos fenólicos, elevada actividade antioxidante e maior efeito na inibição do crescimento de células cancerígenas humanas. As antocianinas foram apontadas como sendo os principais compostos responsáveis pelas bioactividades apresentadas. Contudo, a presença de outros compostos, como os ácidos hidroxicinâmicos, os flavan-3-ois e flavonoides demonstrou ser, também, essencial na protecção ao *stress* oxidativo.

Os resultados obtidos neste estudo serviram de suporte para posterior avaliação da potencial aplicação de tecnologias limpas para o isolamento de ingredientes funcionais a partir dos frutos tradicionais, menos apreciados pelo

consumidor, nomeadamente da maçã *Malápio Fino* e do refugo da cereja *Saco*.

Com o intuito de obter produtos com propriedades anticancerígenas, extractos naturais contendo álcool perilílico foram extraídos da cereja *Saco* utilizando uma extracção fraccionada a alta pressão. A metodologia utilizada consistiu num primeiro passo de extracção com CO_2 supercritico seguido de um segundo passo de extracção onde foram testadas diferentes misturas de CO_2 e etanol. O efeito do fraccionamento e da concentração em etanol no solvente de extracção foram estudados tendo por base o rendimento da extracção, e a concentração em polifenóis e a actividade antioxidante dos extractos obtidos bem como a sua actividade antiproliferativa em células humanas do cancro do cólon.

A fracção obtida a partir da extracção com CO_2 e etanol na proporção 90:10 (v/v) foi a que apresentou maior efeito antiproliferativo, demonstrando ser 150 vezes mais eficiente do que o fruto fresco. Quando comparado com uma droga anticancerígena, o extracto de cereja induziu a paragem do ciclo celular numa fase distinta indicando, desta forma, ser um agente natural promissor em tratamentos de quimioterapia.

Diferentes estratégias de extracção foram ainda estudadas de forma a obter extractos de cereja mais concentrados em compostos com actividade anticancerígena. Deste modo, quando se alterou o grau de pureza dos solventes de extracção foi obtido um extracto com efeito antiproliferativo 16 vezes maior. Além disso, a realização de um passo de extracção prévio à matéria prima, usando metanol ou etanol e água (50:50 v/v), contribuiu também para o aumento da actividade antiproliferativa do produto final.

Por último, foram desenvolvidos concentrados antioxidantes de maçã e de cereja, recorrendo a um processo de adsorção em resinas macroporosas, com vista à sua aplicação como nutracêuticos ou aditivos alimentares. Os produtos obtidos apresentaram cerca de 50% (p/p) em polifenóis e

demonstraram elevado potencial antioxidante em ensaios *in vitro* e em linhas celulares humanas (Caco2 e HepG2).

Por sua vez, estudos de transporte realizados em células Caco2 revelaram que os concentrados de maçã e cereja são biodisponíveis, isto é, são capazes de passar a barreira intestinal de forma a exercerem, posteriormente, o seu efeito benéfico nos vários orgãos e tecidos.

Os resultados obtidos nesta tese mostraram uma elevada bioactividade por parte das variedades tradicionais de maçãs e cerejas que poderá contribuir para a valorização destes produtos e para o desenvolvimento de ingredientes funcionais com potencial aplicação nas industrias alimentar, farmacêutica e cosmética.

THESIS PUBLICATIONS

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Serra, A.T., Matias, A.A., Frade, R.F.M., Duarte, R.O., Feliciano, R.P., Bronze, M.R., Figueira, M.E., de Carvalho, A., Duarte, C.M.M. (2010). Characterization of traditional and exotic apple varieties from Portugal. Part 2- Antioxidant and antiproliferative activities. J Funct Foods, 2, 46-53.

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ABBREVIATIONS

Abbreviation	Full form
AA	antioxidant activity
AAPH	2',2'-Azobis (2-amidinopropane) dihydrochloride
ABTS	2',2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ATCC	American Type Cell Collection
BE	*Bravo de Esmolfe* apple
BSA	bovine serum albumin
C3g	cyanidin-3-glucoside
C3g	cyanidin-3-glucoside
C3r	cyanidin-3-rutinoside
CAA	cellular antioxidant activity
Cac	chlorogenic acid
CAc	chlorogenic acid
Caco2	Caco2 human colon carcinoma cell line
CAE	caffeic acid equivalents
Cat	catechin
CHD	coronary heart diseases
CSE	conventional solvent extraction
CVD	cardiovascular diseases
DAD	diode array detector
DCFH-DA	2',7'-dichlorofluorescin diacetate
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMSO	dimethyl sulphoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dw	dry weight
ECACC	European Collection of Cell Culture
ED	electrochemical detector
ED_{50}	median effective dose
EDTA	ethylenediamine tetraacetic acid
Ep	epicatechin
EPR	electron paramagnetic resonance
ESE	enhanced solvent extraction
EtOH	ethanol
EU	European Union
EVC	*Early Van Compact* cherry

F	*Fuji* apple
FBS	fetal bovine serum
FL	disodium fluorescein
fw	Fresh weight
G	*Golden* apple
GAE	gallic acid equivalents
GG	*Gala Galaxy* apple
Gn	*Garnet* cherry
HBSS	Hank's buffered salt solution
HepG2	HepG2 human hepatocellular carcinoma cell line
HL-60	HL-60 human leukemic cell line
HORAC	hydroxyl radical adverting capacity
HPLC	high performance liquid chromatography
HT29	HT29 human colon cancer cell line
IFEC	Instituto de Formação e Educação Coop
IPATIMUP	Instituto de Patologia e Imunologia Molecular da Universidade do Porto
K3g	kaempferol-3-glucoside
LC-DAD-MS/MS	liquid chromatography with diode array detection mass spectrometry
LDL	low density lipoprotein
Lp	*Lapin* cherry
Ma	*Maring* cherry
MEM	minimum essential medium
MetOH	methanol
MF	*Malápio Fino* apple
MHPE	Multi-step high pressure extraction
MKN45	MKN45 human stomach cancer cell line
Mo	*Morangão* cherry
MS	*Malápio da Serra* apple
MTT	methylthiazolyldiphenyl - tetrazolium bromide
NcAc	Neochlorogenic acid
NCI	National Cancer Institute
NEAA	Non essential aminoacids
ORAC	oxygen radical absorbance capacity
PA	picolinic acid
P_{app}	apparent permeability coefficient
PB1	procyanidin B1

PB2	procyanidin B2
PBS	phosphate buffer solution
PCA	principal component analysis
pCqAc	p-Coumaroylquinic acid
PDO	protected designation of origin
PGI	protected geographical indication
Ph	phloridzin
Pl3r	pelargonidin -3-rutinoside
Pn3g	peonidin-3-glucoside
Pn3r	peonidin-3-rutinoside
POH	perillyl alcohol
PP	*Pêro Pipo* apple
PRC	polyphenol-rich concentrate
Q3g	quercetin-3-glucoside
Q3r	quercetin-3-rhamnoside
RNS	reactive nitrogen species
$ROO^{\bullet}$	peroxyl radicals
ROS	reactive oxygen species
RP	*Reineta Parda* apple
Rut	rutin
S	*Starking* apple
Sc	*Saco* cherry
SE	solvent extract
SFE	supercritical fluid extraction
Sm	*Summit* cherry
SPE	solid phase extraction
TAC	total anthocyanin content
t-BHP	t-butyl hydroperoxide
TEAC	Trolox equivalents antioxidant capacity
TED	total area of chromatogram obtained with electrochemical detection
TEER	transepithelial electrical resistance
TLC	thin layer chromatography
TPC	total polyphenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Ul	*Ulster* cherry
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

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1. DIET AND HEALTH

In recent years, the relationship between the consumption of specific foods and the risk of developing chronic diseases has been extensively discussed in the literature and there is appreciable evidence demonstrating a protective role of diets rich in fruits and vegetables. Eating at least five servings *per* day has been recommended to reduce the risk of cancer and cardiovascular diseases.

1.1. EPIDEMIOLOGIC EVIDENCE

Epidemiological research describes and seeks to explain the distribution of health and disease within human populations. Several studies were performed in order to understand the link between diet and development of chronic diseases; accumulating evidence suggests an inverse association between fruit and vegetables consumption and the incidence of coronary heart diseases (CHD), cardiovascular diseases (CVD) and ischemic stroke (Table 1.1). In particular, results from the Nurses' Health Study and Health Professional's Follow-up Study demonstrate that people with the highest intake of fruits and vegetables (≥8 servings/day) had a relative risk for CHD of 0.80 compared with those consuming less than 3 servings/day. These results are equivalent to a 4% reduction in CHD risk for every 1 serving/day increase in the intake of fruits and vegetables (Joshipura et al., 2001). Green leafy vegetables and vitamin C- rich fruits were identified as the most contributors of the apparent protective effect.

Table 1.1. Association between fruit and vegetables intake and cardiovascular disease, choronary heart disease and stroke: selected epidemiological studies (adapted from Kris-Etherton et al., 2002)

Outcome	Food assessed	Association	Population	Country	Reference
CHD	F and V	-	21930 (M)	Finland	Pietinen et al., 1996
	F and V	-	12763 (M)	7 countries*	Menotti et al., 1999
	F and V	0	3157 (M, W)	Sweden	Rosengren et al., 1999
	V	-	22071 (M)	USA	Liu et al., 2001
	F and V	-	42148 (M) 84251 (W)	USA	Joshipura et al., 2001
	V	-	49 rural counties	China	Zhao and Chen, 2001
	F and V	-	5171 (M) 6669 (W)	USA	Steffen et al., 2003
	F and V	-	501 (M)	USA	Tucker et al., 2005
	F and V	-	194 (M) 96 (W)	Serbia	Nikolic et al., 2008
CVD	F and V	-	39876 (W)	USA	Liu et al., 2000
	F and V	-	9643 (M, W)	USA	Bazzano et al., 2001
	F and V	-	9606 (M, W)	USA	Bazzano et al., 2002
Stroke	F and V	-	832 (M)	USA	Gillman et al., 1995
	F and V	- (V only)	38683 (M) 75596 (W)	USA	Joshipura et al., 1999
	V	-	49 rural counties	China	Zhao and Chen, 2001
	F and V	0	5171 (M) 6669 (W)	USA	Steffen et al., 2003

-, inverse association; 0, no association; CHD, coronary heart disease; CVD, cardiovascular disease; F, fruits; V, vegetables; M, men; W, women

* USA, Finland, Netherlands, Italy, former Yugoslavia, Greece and Japan

For cancer, more than 200 epidemiological studies have been performed but the findings are not conclusive. Despite that, the 2007 report Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global perspective by the WCRF-AICR* indicate a probable protective role of fruit, vegetables and their constituents against some types of cancer (Table 1.2).

* World Cancer Research Found and American Institute for Cancer Research

Table 1.2. Summary of the WCRF-AICR report on the possible effect of high fruit and vegetable consumption on cancer risk

Cancer site	Fruits	Vegetables
Mouth, pharynx and larynx	Probable	Probable
Nasopharynx	Limited- suggestive	Limited- suggestive
Oesophagus	Probable	Probable
Lung	Probable	Limited- suggestive
Stomach	Probable	Probable
Pancreas	Limited- suggestive	Limited- no conclusion
Liver	Limited- suggestive	Limited- no conclusion
Colon and rectum	Limited- suggestive	Limited- suggestive
Breast	Limited- no conclusion	Limited- no conclusion
Ovary	Limited- no conclusion	Limited- suggestive
Endometrium	Limited- no conclusion	Limited- suggestive
Cervix	Limited- no conclusion	Limited- no conclusion
Prostate	Limited- no conclusion	Probable
Kidney	Limited- no conclusion	Limited- no conclusion
Bladder	Limited- no conclusion	Limited- no conclusion
Skin	Limited- no conclusion	Limited- no conclusion
Thyroid	Limited- no conclusion	Possible

The health promoting effect of fruit and vegetables is related with their bioactive constituents, in particular phenolic compounds. These substances act through several mechanisms, such as reducing oxidative stress, improving lipoprotein profile, lowering blood pressure and improving homeostasis regulation thus contributing to a healthy lifestyle (Scalbert et al., 2005).

1.2. PHYTOCHEMICALS AND THEIR BIOACTIVITIY

Phytochemicals are bioactive compounds, present in fruit, vegetables and other plant foods. It is estimated that more than 5000 individual phytochemicals have been identified and are classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulfur

compounds (Figure 1.1) (Liu, 2004). Among all, phenolic are together with and carotenoids the most studied phytochemicals.

Phenolics, or polyphenols, are characterized structurally by the presence of one or more aromatic rings with one or more hydroxyl groups. According to their chemical structure, phenolics can be divided into several classes, namely phenolic acids, flavonoids, stilbenes, coumarins and tannins (Figure 1.1).

Phenolics are the products of secondary metabolism of plants, providing essential functions in their physiology, being involved in their morphology (colour and mechanical support), growth (phenolic acids have been related to nutrient uptake, protein synthesis, enzyme activity and photosynthesis), reproduction (attracting birds and insects that helping pollination) and in protection against attack by pathogens or other stress factor (Gould and Lister, 2006). In diet, they are the major responsible of health promoting effect. The bioactivity of polyphenols has been, to some extent, associated to their antioxidant properties, i.e., capacity to scavenge free-radicals. Free radicals, such as reactive oxygen species (ROS), are continuously produced in the body, as by-products of metabolic activity, but can also be derived from exogenous sources (cigarette smoking, pollution and physical exercise). When present in higher amounts in cells, ROS can cause oxidative damage to large biomolecules such as lipids, proteins and DNA, being involved in the onset development of many chronic degenerative diseases. The current knowledge related with potential health benefits of polyphenols was recently reviewed by Stevenson and Hurst (2007) (Table 1.3).

There is an evidence that polyphenols could acts as “indirect” antioxidants through induction of endogenous protective enzymes, namely superoxide dismutase, catalase and glutathione peroxidase (Zhan and Yang, 2006). The potential of these phenolics to mediate the regulation of cellular processes such as inflammation has also been reported (Huang et al., 2006).

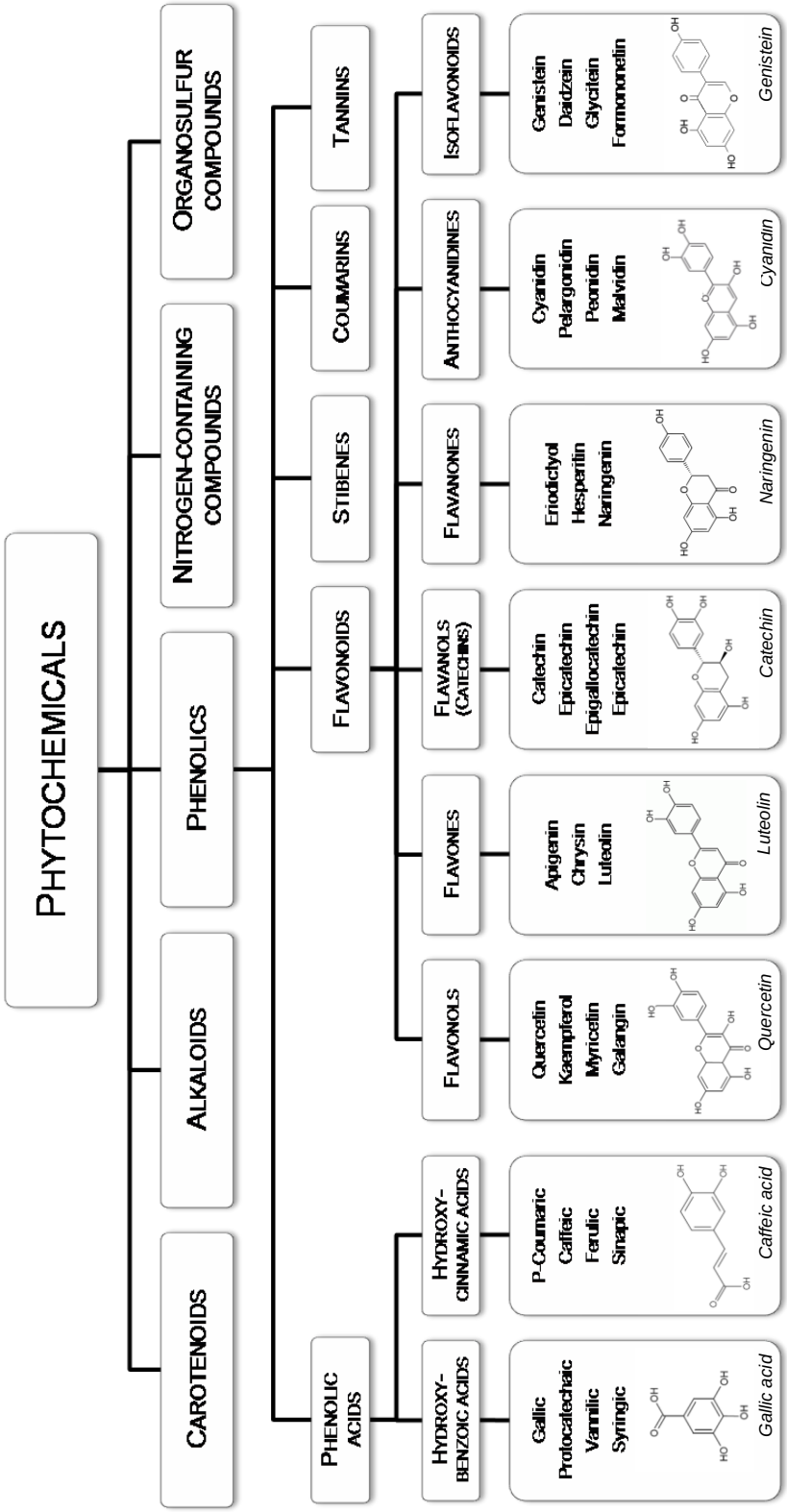

Figure 1.1. Classification of dietary phytochemicals (adapted from Liu, 2004)

Table 1.3. Summary of main current findings about phenolics and their potential health benefits (adapted from Stevenson and Hurst, 2007)

Finding
• *In vitro*, phenolic can induce the oxidant enzymes superoxide dismutase, catalase and glutathione peroxidase.
• Polyphenols appear to be pro-oxidants in cell-based studies, but nevertheless appear to protect cultured cells from oxidative stress.
• Human trials show correlation between polyphenols, in particular flavonoids, intake and reduce incidence of cancer and cardiovascular disease, and slower neuro-degeneration.
• In vitro regulation of inflammatory pathways by inhibition of signalling molecules such as TNF and by down-regulation of inflammatory genes be the mechanism of the beneficial effect s on CVD.
• Polyphenols may be able to help regulate blood pressure.
• Polyphenols may be able to modulate human responses in allergic conditions.
• Regulation of cell proliferation and differentiation, angiogenesis and apoptosis; may explain anti-cancer effects.
• Neuro-protection, by protecting neuronal cells from oxidative stress, induction of antioxidant defences and modulation of signalling cascade and apoptotic process.
• Regulation of metabolic syndrome, via inhibition of glucose uptake and regulation of some metabolic pathways.
• Benefits to gut health by growth inhibition of pathogenic gut bacteria and modulation of inflammatory bowel conditions.

Dietary intake of polyphenols is estimated to be approximately 1g/day (Clifford, 2004). Only 10% are absorbed upper gastrointestinal track by passive diffusion and the remainder pass to colon being metabolized into bioavailable phenolic acids (Aura et al., 2002; Jenner et al., 2005). Figure 1.2 shows a schematic representation of the main processes by which polyphenols mediate health benefits.

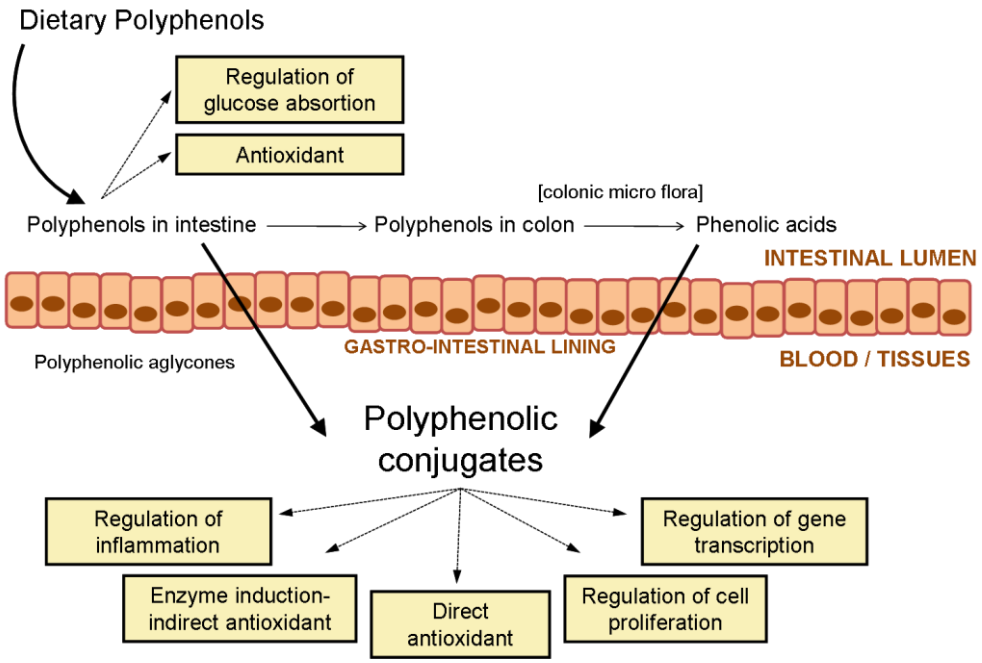

Figure 1.2. Schematic representation of the main processes by which phenolics mediate health benefits (adapted from Stevenson and Hurst, 2007)

It is important to note that phenolic compounds are less potent than pharmaceutical drugs, but since they are ingested regularly in significant amounts as part of the diet, they may have a noticeable long-term physiological effect (Espin et al., 2007).

1.3. APPLES AND CHERRIES

Apples (*Malus domestica*) and sweet cherries (*Prunus avium*) are very attractive fruits for consumers due to their taste, sweetness and wealth of nutrients being easily adapted to a regular diet. These fruits constitute rich source of phytochemicals, containing more polyphenols than other commonly consumed, such as pear, orange, peach, banana, pineapple and watermelon (Figure 1.3).

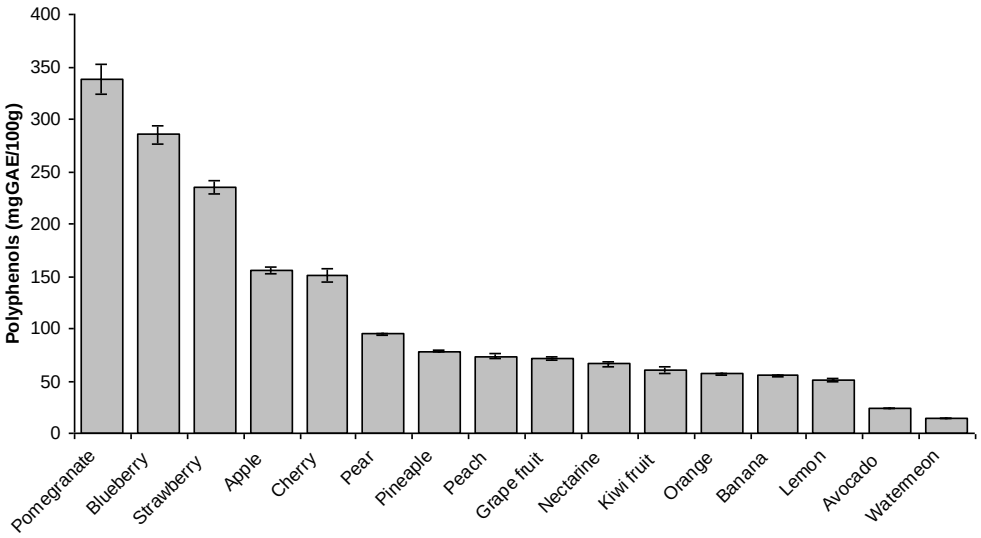

Figure 1.3. Total phenolic content of selected fruits (Wolfe et al., 2008)

The major compounds identified in apple are flavonoids and phenolics acids (Table 1.4) which includes quercetin glycosides, procyanidins, catechin, epicatechin, chlorogenic acid and phloridzin (Lee et al., 2003) and their concentrations are higher in the apple peel than in the flesh (Escarpa and Gonzalez, 1998). For instance, quercetin conjugates are found exclusively in the peels.

Cherry polyphenols include flavonoids, hydroxycinnamic acids and hydroxybenzoic acids (Gao and Mazza, 1995; Gonçalves et al., 2004) (Table 1.4). Among all, special interest has been shown for anthocyanins, which are the compounds responsible for the red colour of fruits, due to their strong antioxidant and anti-inflammatory activities (Wang et al., 1999).

The concentrations of these polyphenols may depend on many factors, such as fruit cultivar, maturation stage, climatic conditions, storage and processing (Awad et al., 2001; Gonçalves et al., 2004; Kallay et al., 2008; Kyrakosyan et al., 2009; Lata et al., 2009; Serrano et al., 2005; Stracke et al., 2009; van der Sluis et al., 2001; Vrhovsek et al., 2004).

Table 1.4. Polyphenolic compounds identified in apples and sweet cherries.

APPLES	CHERRIES
4-Coumaroylquinic acid; Caffeic acid; Catechin; Chlorogenic acid; p- Coumaric acid; Cyanidin-3-galactoside; Epicatechin; Ferulic acid; Gallic acid; Kaempferol; Kaempferol glucosides; Phloretin; Phloretin glucosides; Phlorizin; Procyanidin B1; Procyanidin B2; Procyanidin C1; Quercetin glucosides; Quercetin;	Catechin; Chlorogenic acid; Cinnamic acid; p-Coumaroylquinic acid; Cyanidin-3-glucoside; Cyanidin-3-rutinoside; Ellagic acid; Epicatechin; Ferulic acid; Gallic acid; Gentisic acid; Kampferol glucosides; Neochlorogenic acid; Pelargonidin-3-rutinoside; Peonidin-3-glucoside; Peonidin-3-rutinoside; Quercetin; Rutin; Salicylic acid; p-Xydroxybenzoic

(Gonçalves et al., 2004; Jakobek et al., 2009; Lee et al., 2003; Tanchev et al., 2006; Usenik et al., 2008; Zessner et al., 2008)

In the last years, human, animal and cell culture studies show that there is an association between the phenolic compounds found within apples/cherries and a wide variety of effects that may help prevent chronic diseases

In particular, epidemiological studies have linked the consumption of apples with the reduced risk of some cancers (Arts et al., 2001; Feskanich et al., 2000; Hertog et al., 1994; Knekt et al., 1997; Le Marchand et al., 2000), thrombotic stroke (Knekt et al., 2000), ischemic heart disease (Arts et al., 2001), lung dysfunctions (Butland et al., 2000), asthma (Shaheen et al., 2001) and type-2 diabetes (Knekt et al., 2002). Additionally, *in vitro* and animal studies have demonstrated that apples have high antioxidant activity (Lata, 2007; Lee et al., 2003; Tsao et al., 2005), can inhibit cancer cell proliferation (Eberhardt et al., 2000; Veeriah et al., 2006; Yoshiawa et al., 2005) decrease lipid oxidation (Pearson et al., 1999) and lower cholesterol (Aprikian et al., 2001).

Concerning cherries, epidemiological evidence suggests that the consumption of anthocyanins reduces the risk of cardiovascular diseases (Artalejo et al., 1997), diabetes (Jayaprakasam et al., 2005) and cancer (Rossi et al., 2007). Additionally, human studies demonstrated cherries' anti-inflammatory potential (Kelley et al., 2005) and anti-gut efficiency (Jacob et al., 2003). Antioxidant, anti-inflammatory and anticancer activity of cherries have been also explored

in animal studies and *in vitro* assays (Kang et al., 2003; Seeram et al., 2001; Usenik et al., 2008).

Overall, the potential benefits of apples and cherries are numerous. Regular consumption of these fruits, as a part of a healthy diet, may aid in the prevention of chronic diseases and maintenance of good health.

2. NUTRACEUTICALS AND FUNCTIONAL FOODS

Nowadays food is functionalized and tailor made for specific groups (e.g. athletes, the elderly, and people with specific conditions). Increased life expectancy has resulted in an increase in the quest for diets which allow for a healthy ageing.

In the 1990's nutraceuticals appeared in the market. These are pharmaceutical forms (pills, capsules, powders) containing food bioactive compounds as active principles. Among all, natural phytochemicals have become a very significant source for nutraceutical ingredients.

In recent years, special interest has been focus on recovery of these compounds from food industry by-products in an effort to find value-added applications for these wastes. In order to obtain such valuable compounds extraction techniques have been widely investigated. Traditional methods, such as conventional solvent extraction and Soxhlet extraction, have several drawbacks; they are time consuming, laborious, have low selectivity and/or low extraction yields. Moreover, these traditional techniques employ large amounts of toxic solvents (Luque de Castro and Garcia-Ayuso, 1998). At present extraction methods able to overcome the above-mentioned problems are being studied. Among all, high pressure extraction has becoming a very promising technology (King, 2000). Additionally, adsorption processes for the purification and fractionation of crude plant extracts may be helpful for the production of tailor-made purified phytochemical preparations (Kammerer and Carle, 2007).

2.1. HIGH PRESSURE EXTRACTION

High pressure technology has been recognized to be a promising process for the extraction of bioactive compounds. This technology utilizes solvents such as hexane, light petroleum, ethanol and water (Herrero et al., 2004) at temperatures above their boiling point, under high pressures in order to increase the extraction efficiency with respect to extraction time, solvent consumption and extraction yields. This is accomplished through improved solubility and mass transfer effects (Richter et al., 1996).

Alternatively, high pressure carbon dioxide (CO_2) has also been used. CO_2 is described as a “green solvent” and has been explored under either subcritical or supercritical conditions in a widely known process designated supercritical fluid extraction (SFE).

A pure component is considered to be in a supercritical state if its temperature and pressure are higher than the critical values (T_c and P_c, respectively) (Figure 1.4).

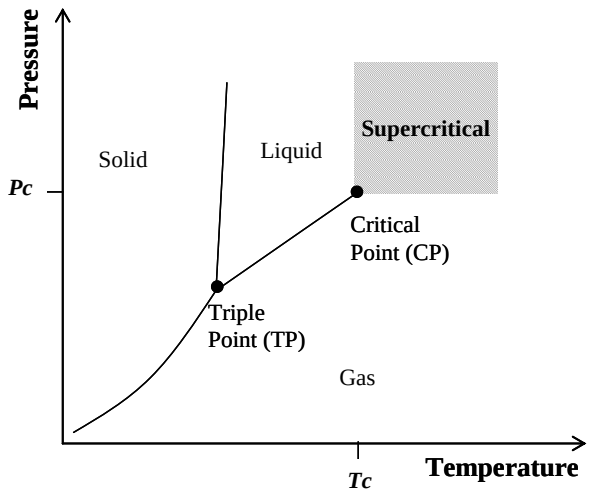

Figure 1.4. Definition of supercritical state for a pure component

The most important feature of a supercritical fluid is that in the supercritical region, minor variations of pressure or temperature lead to a significant variation of the solvent density and consequent solvent power. There are a lot of examples in the literature where the adjustment of operating conditions is used to change the selectivity of CO_2. In a recent example Ruiz-Rodriguez et al. (2008), reported the use of high pressure CO_2 in the extraction of solanesol and nicotine from tobacco leaves. The authors observed that the solanesol/nicotine ratio is very sensitive to extraction temperature and conclude that it is possible to use near critical carbon dioxide to obtain an extract enriched in solanesol when working at low temperatures.

PRACTICAL ISSUES OF SFE

The efficiency of supercritical fluid extraction is dependent by a number of factors related with pre-treatment of sample, extraction process and trapping conditions.

Particle size plays an important role in the extraction process. Smaller particle sizes increases external surface area for extraction which results in higher extraction yields and qualities of the extraction product. However, if particles are too small they can cause problems of channelling inside the extraction bed, leading to a loss in efficiency and yield of process (Reverchon and De Marco, 2006).

Selection of supercritical fluid is crucial for the development of a supercritical fluid process. Carbon dioxide (CO_2) is the most frequently used solvent because it is readily available, relatively cheap, non toxic and non-inflammable. It allows supercritical operations at relatively low pressures and temperatures (P_c= 7.38 MPa; T_c= 31.2 ºC) being suitable for extracting heat labile natural compounds. Moreover, the extraction system provides a light- and oxygen-free environment, thereby minimizing degradation and preserving the bioactive and antioxidant properties of extracts. Concerning this, extracts from supercritical treatments with CO_2 can be regarded as all natural, and the

products allowed for food applications have the GRAS† status. However, the main drawbacks of CO_2 are its nonpolar and lipophilic nature and its inability to extract compounds of high molecular weight. For this reason, the use of suitable co-solvents has been proposed to enhance the solubility of the target compounds and/or to increase the extraction selectivity. The common modifiers used are alcohols (methanol and ethanol), water, chloroform and ethyl acetate, and their amount should be reduced in order to minimize the volume of hazardous solvents in the process. Among all modifiers, ethanol and water are the only acceptable for food and pharmaceutical applications. More recently, natural lypophilic solvents such as vegetable oils (olive oil, hazelnut oil and soybean oil) have been used as co-solvents (Krichnavaruk et al., 2008; Shi et al., 2009; Vasapollo et al., 2004).

The selection of operating conditions depends on the specific compound or family of compounds to be extracted. The most relevant process parameters are the extraction pressure and temperature, which can be used to tune the selectivity of supercritical fluid. Increasing the pressure at constant temperature increases the density of the solvent and therefore its solvating power (Taylor, 1996) resulting in a higher extraction yields. However, it could promote the extraction of unwanted compounds, reducing selectivity (Macias-Sanchez et al., 2005; Ziemons et al., 2005). At a constant pressure, the increase of temperature reduces the density of supercritical CO_2 and consequently decreases the solvent power of the supercritical solvent resulting in lower yields (Taylor, 1996). On the other hand, it increases the vapour pressure of the compounds enhancing their extraction. For recovery of thermolabile compounds, temperature should be fixed between 35 and 60ºC.

Trapping is often the source of lower extraction yields and it depends on the sample matrix, compounds extracted and extraction conditions (Cheah et al., 2006). In general, there are three forms of collection: solid trapping, liquid trapping and by sublimation.

† Generally recognized as safe

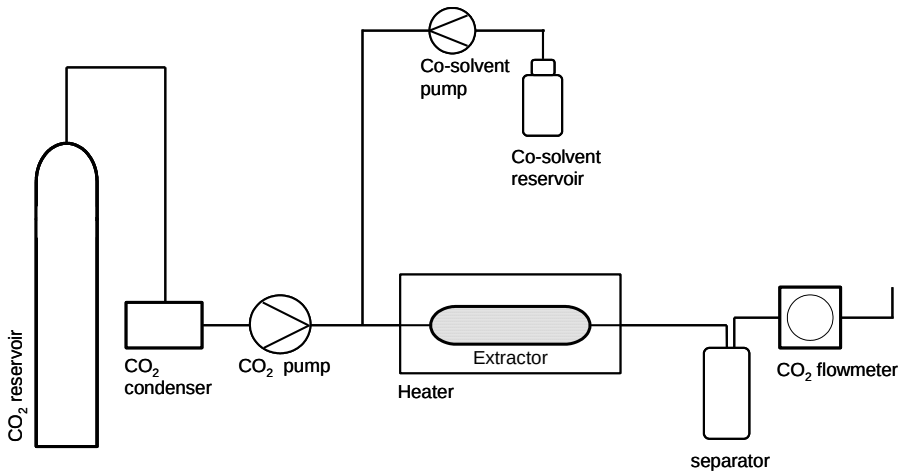

Figure 1.5. Schematic diagram of a process-scale supercritical fluid extraction system

A supercritical fluid extraction system is shown in figure 1.5. Basically, it consists of a solvent pump, which delivers the fluid throughout the system, a modifier pump (in the case of extractions using a co-solvent), an extraction cell, commonly equipped with control of temperature and pressure, and a separator in which the extract is collected and the solvent depressurized.

ADVANTAGES AND DISADVANTAGES OF SFE

The significant benefits of supercritical fluids include their liquid-like densities and solvating power. By changing the extraction pressure and temperature, the density of the solvent is modified and therefore it is possible to fine-tune the selectivity. Moreover CO_2 is a gas at room temperature, so once the extraction is completed and the system decompressed, a substantial elimination of solvent is achieved without residues. This results in a solvent free extract.

In comparison with conventional methodologies SFE is more precise and selective allowing the use of multi-stage extraction processes which enriches

the concentration of the compound of interest. Additionally, as SFE extraction does not use or uses only minimal amounts of organic solvents, it is a more environmentally friendly technique. Moreover, supercritical fluid extracts do not require additional sterilization stages since bacteria and other living organisms can be inactivated with high-pressure gradient during pressure release (Spilimbergo et al., 2002; Spilimbergo et al., 2003).

However, the higher investment costs and the onerous operating conditions of the process have hindered a wider development. To overcome this drawback and make costs of supercritical fluid extraction processes competitive, it is essential that the product to be extracted has a recognized high added value and presents a much better quality than using conventional extraction techniques.

APPLICATIONS OF SFE

Supercritical fluid extraction has been widely used to isolate flavours, essential oils, seed oils, antioxidants and bioactive principles. Some of those applications were extensively reviewed (Catchpole et al., 2009; Cheah et al., 2006; Diaz-Reinoso et al., 2006; Reverchon and De Marco, 2006; Sahena et al., 2009) and are summarized in table 1.6. In particular, the recovery of high added value compounds from food industry by-products, such as polyphenols from grape and fruit pomaces (Adil et al., 2007; Adil et al., 2008; Casas et al., 2010), lycopene from tomato industrial waste (Nobre et al., 2009) and tocopherol from by-products of olive oil industry (Ibanez et al., 2000), was also explored by supercritical fluid technology.

Table 1.5. SFE of oils and high added value compounds

Extraction	Solute	Sample
Oils	*Essential oils*	Bacuri fruit shells; Basil leaves; Chamomile flowers; Clove bud; Eucalyptus leaves; Ginger; Hiproise fruit; Laurel leaves; Lemon balm; Lemon bergamot; Lemon eucalyptus; Lemongrass leaves; *Lippia alba;* Lovage leaves and roots; Marjoram leaves; Mint leaves; Oregano; Pennyroyal; Pepper black; Sage leaves; Spiked thyme; Star anise; Thyme; Tuberose concrete
	Seed oils	Black cumin seeds; Blackcurrant seed; Borage seeds; Celery roots; Coriander seeds; Evening primrose seed; Fennel seeds; Grape seeds; Hemp seed; Hiprose seeds; Kiwifruit seed; Medlar seeds; Palm kernel oil; Rosehip seed; Vernonia seeds
High added value compounds	*Polyphenols*	Aloe vera leaf skin; Apple pomace; Baical skullcap root; Boldo; Bushy lippia; Cashew nut shell; Chamomile; Chardonnary grape seeds; Chinese skullcap, root; Corinder seeds; Echinacea; Elderberry; Eucalyptus leaves; Fennel seeds; Ginger; Ginkgo; Ginseng root hair; Grape seeds and skin; Green tea; Guaco, leaves; Hawthrn, flower; Helichrysum dried flowers head; Hiprose; Hop; Hu zang roots; Hyperici grains, leaves and flowers; Lemon balm; Magnolia; Marigold, flower; Maritime pine bark; Marjoram; Nigella seeds; Olive leaves; Osage orange tree; Paeonia cortex; Passiflora leaves; Peach pomace; Pepper oleoresin, black; Pistachio hulls; Propolis; Red grape pomace; Rosemary; Sage; Savory oil; Schisandra black seeds, leaves and fruits; Sea buckthorn; Sesame seed, black; Sour cherry pomace; Soybean flower; St. John's wort; Summer savory; Sweet gale fruits; Sweet grass; T. catappa seeds and leaves; Tamarind, seed coat; Thyme; Turmeric; Vanilla beans; Water soaked
	Carotenoids	Alfalfa leaf; Appricot pomace; Buriti fruit; Carrot; Crustaceans; Krill; Marjoram; Microalgae (Chlorella vulgaris, Nannochloropsis gaditana, Spirulina maxima, Dunalilella salina, Haematococcus pluvialis, Phaffia rhodozyma); Paprika; Stinging nettle; Sweet potato; Tomato and tomato by products (paste waste, skin)
	Tocopherols	Aloe vera leaves; Coriander seeds; Dill; Espinheira Santa; Fresh bay; Milk thistle; Olive oil industry by-products; Olive tree leaves; Parsley; Rice; Sesame seed, black; *Silybum marianum*; Soybean flakes; Soybean oil by-product; Spearmint; Wheat germ
	Squalene	Espinheira Santa; Indian almond leaves; Olive oil industry by-products; Shark liver oil; Tropical almond leaves and seeds
	Fatty acids	Brown seaweed; Cardamom seed; Corn bran; Cotton seed; *Cunninghamella echinulata*; Grape seeds; Ground beef; Mackerel; Oat Bran; Paenuts; Palm kernel; Pecan ; Pistachio; Pythium rregulary ; Rapeseed; Rice bran; Rosehip sedd; Safflower; Sardine oil; Saw Palmetto berries; Soybean ; Sunflower; Wheat germ

2.2. ADSORPTION TECHNOLOGY

Adsorption technology is a commonly applied process to recover bioactive compounds from plant materials and consists on a solid phase extraction (SPE).

The principles of SPE involve a partitioning of compounds between two phases. The analytes to be extracted are partitioned between a solid and a liquid phase and these analytes must have a greater affinity for the solid phase than for the sample matrix (retention or adsorption step). Compounds retained on the solid phase can be removed at a later stage by eluting with a solvent with a higher affinity for the analytes (elution or desorption step).

The different retention and elution mechanisms are due to intermolecular forces between the analyte, the active sites on the surface of the solid phase and the liquid phase or matrix (Berrueta et al., 1995).

The experimental procedure consists in five steps:

1- Activation of solid phase by passing trough it an appropriate solvent that conditions its surface.

2- Replacing the activation solvent with a liquid similar in composition to the sample matrix.

3- Application of sample; the analytes will be retained by the solid phase (adsorption or retention step).

4- Removal of interfering compounds retained in step 3 with a solvent that does not remove the analytes (washing step)

5- Elution of the analytes from the solid phase with an appropriate solvent (desorption or elution step)

Adsorption processes are often developed empirically and successfully applied for a large scale purification and concentration of bioactive compounds (Di Mauro et al., 1999; 2000; 2002). However, the mechanisms behind the adsorption of components from complex mixtures still remain unknown.

PRACTICAL ISSUES

In order to assure a complete recovery of product and impurities, the material and the adsorption/elution steps should be optimized.

The selection of adsorbents is critical for the development of an adsorption process. Adsorbent characteristics, such as particle size, surface, area and porosity should be taken in consideration in each case study.

For the purification of polyphenols various adsorbents have been used. One of the most commonly applied is a polymeric resin Amberlite® XAD16 (Figure 1.6) (Nair, 2004; Weinand and Dedardel, 1994).

The adsorption capacity of an adsorbent for a solute may vary with processing conditions such as temperature and pH value of solution. Additionally, in complex mixtures the absorption behaviour of each individual compound should be taken in consideration as well as the presence of other co-solutes that can also interact with the adsorbent (Kammerer et al., 2010).

The elution step is usually performed with alcohols, in particular ethanol as it is acceptable for food and pharmaceutical applications.

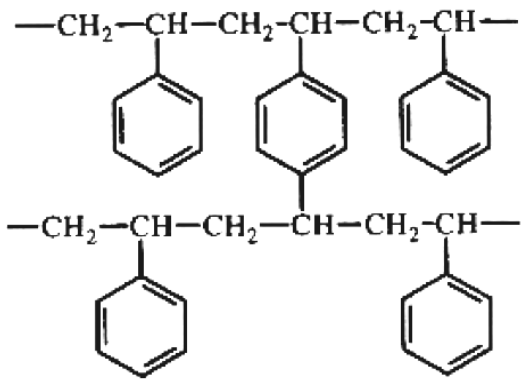

Figure 1.6. Chemical structure of Amberlite® XAD 16. (XAD 16 is a macroreticular and non-polar styrene-divinilbenzene resin that adsorbs and releases ionic species through hydrophobic and polar interactions. The adsorption capacity is due to Van der Waals forces)

Finally, adsorption processes can be performed in a batch mode, by putting into a vessel the liquid phase containing the analyte with the adsorbent material and shaking for a controlled time (Silva et al., 2007), or in continuous, where the adsorbent is packet into a column and liquid phase pass through it by suction or by positive pressure (Zessner et al., 2008).

ADVANTAGES AND DISADVANTAGES

Adsorption technology is one of the most commonly applied processes for the recovery of polyphenols from plant crude extracts and is gaining increasing importance in the food industry (Kammerer and Carle, 2007; Scordino et al., 2005). The application of synthetic resins has several advantages, such as relatively low operation costs, simple handling, high total adsorption capacities for a large number of compounds and easy regeneration (Kammerer et al., 2010). Moreover, the use of resins for food production proposes is regulated by the US Food and Drug Administration and the Council of Europe.

Even though these procedures are commonly applied and are part of patented process (Garti et al., 2003; Shrikhande, 1983; Wood et al., 2008) systematic data concerning adsorption and desorption of compounds and recovery rates are still scarce.

APPLICATIONS

Adsorption technology has long been used for food processing, such as the debittering of fruit juices (Manlan et al., 1990), for the removal of browning reaction products from apple juice (Carabasa et al., 1998; Gokmen and Serpen, 2002) or other unwanted compounds from olive oil residue (Ferreira-Dias et al., 2002).

Table 1.6. Phytochemicals recovery from plant sources using adsorption process

Compound	Sample	Adsorbent	Reference
Polyphenols	Apple juice	Amberlite XAD16	(Zessner et al., 2008)
		Alimentech P-495	(Kammerer and Carle, 2007)
	Olive oil mill waste water	Amberlite XAD4, XAD16 and XAD17HP	(Agalias et al., 2007)
	Inga edulis leaves	Amberlite XAD7and XAD16 Relite EXA-90HP, EXA-118	(Silva et al., 2007)
	Hypericum perforatum	LSA resin	(Zou et al., 2004)
	Spinach	Amberlite® XAD16HP, XAD17HP	(Aehle et al., 2004)
	Orange juice waste water	Kastell S 112	(Di Mauro et al., 2000)
	Orange peel	Kastell S 112	(Di Mauro et al., 1999)
Anthocyanins	Tart cherry	Amberlite XAD16	(Bobe et al., 2006)
	Grape pomace	Amberlite XAD16	(Kammerer et al., 2005)
	Blood orange (by- product of juice industry)	Relite EXA 90	(Scordino et al., 2005)
		Sepabeads SP70, Relite EXA90	(Di Mauro et al., 2002)
	Black chokeberry	Serdolit PADIV, Amberlite XAD7	(Kraemer-Schafhalter et al., 1998)

Furthermore, this process has already been demonstrated to be applicable for the selective recovery of high-value compounds, such as polyphenols and anthocyanins, from several plant sources (Table 1.6).

3. SCOPE AND STRUCTURE OF THE THESIS

As mentioned previously, apples and cherries constitute rich sources of functional ingredients, including polyphenols.

In Portugal, approximately 300,000 tons of apples and 17,000 ton of cherries were produced per year. Among all varieties, there are some traditional fruits which are only cultivated in specific regions of the country. In particular, *Bravo de Esmolfe* apple and *Saco* cherry have protected designation of origin (PDO) and protected geographical indication (PGI) registration according to the EU regulations, respectively. These fruits are only produced in the region of "Beiras" and are very appreciated by consumers due to their unique organoleptic properties. However, they are less produced and more expensive than the exotic cultivars.

The aim of this thesis, schematically presented in figure 1.7, is to valorize these traditional Portuguese fruits through the evaluation of their biological activity and the development of functional ingredients, in order to increase their productivity and competition in the market.

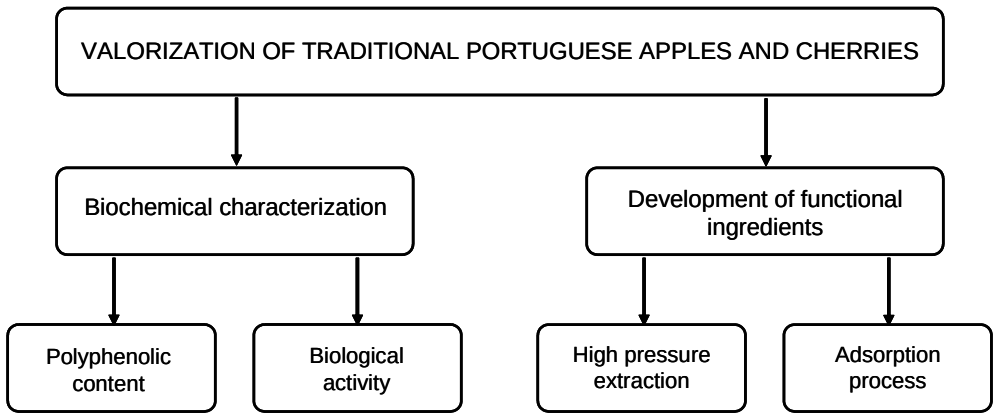

Figure 1.7. Thesis rational

The present chapter gives a general overview of the health benefits of fruits, with a special focus on apples and cherries. Moreover, an introduction of clean and environmentally friendly extraction technologies for the development of functional products are also included.

The other chapters are organized as follows:

Chapter 2- Identification of bioactive response in traditional Portuguese fruits

In this chapter, traditional apples and cherries are evaluated for their bioactive effect. Different methodologies were performed and fruits were characterized in terms of phenolic content, antioxidant activity and antiproliferative effect against human colon and gastric cancer cells.

In Part I, a total of 26 apples, including exotic and traditional varieties, was screened for polyphenolic content and antioxidant activity. From this analysis four promising traditional apples, namely *Bravo de Esmolfe*, *Malápio Fino*, *Malápio da Serra* and *Pêro Pipo*, were selected and fully characterized. Results were further compared with five exotic cultivars (*Fuji*, *Gala Galaxy*, *Golden*, *Reineta Parda* and *Starking*). A two-year comparison of the phenolic content and bioactivity of the nine apples was also included in this Part.

In Part II, the bioactivity of two traditional cherry cultivars (*Saco* and *Morangão*) was studied and compared with seven exotic varieties (*Summit*, *Maring*, *Van*, *Early Van Compact*, *Lapin*, *Ulster* and *Garnet*).

In both parts the data obtained from the phenolic characterization was correlated with biological activity in order to understand which compound(s) is(are) responsible for the antioxidant and antiproliferative activity of fruits.

Chapter 3- Development of functional ingredients

New functional ingredients obtained by clean separation processes from rejected traditional fruits (fruits that do not fit all the requisitions to be sold as

fresh edible products) are presented in this chapter. *Saco* cherry culls and *Malápio Fino* apples were used as raw material due to their recognized rich content in bioactive compounds.

In Part I, fractionated high pressure extraction, performed with CO_2 plus CO_2:ethanol mixtures, was explored in order to develop a natural anticancer extract from cherries. The influence of processing parameters such as ethanol concentration in solvent mixture and the effect of fractionation was studied. At the end, the anticancer activity of cherry extracts was evaluated using a human colon cancer cell model and results were compared with doxorubicin, a common drug used in chemotherapy. Still in this Part, processing strategies were adopted in an effort to further increase the antiproliferative potential of cherry extract.

In Part II, the recovery of powerful antioxidant concentrates from apples and cherries using adsorption in macroporous resins is described. The fruit extracts were characterized in terms of phenolic composition and antioxidant activity using cell-free and cell-based assays. Transport studies in Caco2 cell model were also included in order to study the bioavailability of extracts.

Chapter 4- General discussion and final conclusions

This chapter encloses an overall discussion and major conclusions of this thesis.

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CHAPTER 2

IDENTIFICATION OF BIOACTIVE RESPONSE IN TRADITIONAL PORTUGUESE FRUITS

As reported previously, apples and cherries are good sources of polyphenols. The concentration of these bioactive compounds varies during fruit maturation, ripening stage, growing season, storage conditions and, most importantly, between different varieties.

In this chapter, traditional Portuguese apples and cherries were characterized in terms of phenolic composition, antioxidant potential in both cell-free and cell-based assays and antiproliferative effect using human cancer cells. Results were further compared with exotic cultivars.

PART I

APPLES

Results of this part were published in international scientific journals and books:

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ABSTRACT

Apples are known to have many health-promoting activities, especially anticancer and antioxidant effects. Most of these actions are believed to be due to their polyphenolic content which varies greatly between different varieties.

In this work, 26 apples produced in Portugal were analysed for their polyphenolic composition and biological effect. Two traditional varieties, namely *Bravo de Esmolfe* and *Malápio Fino*, demonstrated to be rich sources of functional ingredients with powerful antioxidant activity and relevant antiproliferative effect against human cancer cells. The exotic *Reineta Parda* was the best cultivar in inhibiting human gastric and colon cancer cell proliferation. The correlation between phenolic composition and bioactivity demonstrated that catechin, epicatechin and procyanidin B1 are the major contributors of the antioxidant activity of apples. Additionally, epicatechin and procyanidin B1 together with procyanidin B2 and phloridzin play an important role against human cancer cell proliferation.

The results obtained in two different crop years demonstrated that climatic conditions, in particular precipitation, influence phenolic content of apples and, consequently, their antioxidant and antiproliferative effect. Nevertheless, the major differences in biological activity occur within different varieties.

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1. INTRODUCTION

Bravo de Esmolfe, a traditional Portuguese apple, is only produced in a restricted and small inland region in northern of Portugal, namely "Beiras". This apple was recognized by classification of "Protected Designation of Origin" product (Diário da República, 1994) and due to its peculiar sensory properties it has been cultivated more and more by the farmers.

In the last few years, *Bravo de Esmolfe* has doubled in price compared to exotic varieties such as *Golden* and *Starking*. Despite the fact that it is appreciated by consumers its annual production does not exceed 1500 tons which is insufficient for market demand. Moreover, there is no reported data concerning phytochemical and bioactive characterization of these apples making this study quite relevant for providing agriculture and food industries with experimental data.

The main aim of this work was to evaluate the bioactive effect of *Bravo de Esmolfe* and others traditional Portuguese varieties which had high commercial expression in the 50s, such as *Malápios* and *Camoesas*, in an effort to distinguish promising functional apples and also to contribute for the fruit growing development in specific regions of the country. For this purpose, 26 apples from Beiras and Minho, including 18 traditional Portuguese varieties (*Bravo de Esmolfe*, *Malápio Fino*, *Malápio da Serra*, *Pêro Pipo*, *Camoesa*, *Malápio da Ponte*, *Pardo Lindo*, *Pipo de Basto*, *Porta da Loja*, *Costa*, *Maçã Pedra, Maçã Verdeal*, *Malápio do Jordão*, *Malápio IFEC*, *Pêro da Lixa*, *Pêro de Coura*, *Pêro Rei* and *Tromba de Boi*) and 8 exotic cultivars (*Golden*, *Starking*, *Fuji*, *Gala Galaxy*, *Reineta Parda*, *JF13*, *JF29* and *Tarte*) were studied and the most promising apples were selected taking into account their polyphenolic composition, antioxidant potential and antiproliferative effect in human cancer cells.

2. EXPERIMENTAL PROCEDURE

2.1. MATERIALS

Acetone, 2',2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), bovine serum albumin (BSA), bradford reagent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), caffeic acid, cobalt floride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) methylthiazolyldiphenyl - tetrazolium bromide (MTT), picolinic acid (PA), phosphate buffer solution (PBS), t-butyl hydroperoxide (t-BHP) and sodium carbonate were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Folin Ciocalteau reagent was acquired from Panreac (Barcelona, Spain). Disodium fluorescein (FL) was obtained from TCI Europe (Antwerp, Belgium) and ferrous sulphate- $FeSO_4$ - was from Merck (Darmstadt, Germany). Human low density lipoprotein (LDL) was purchased from Calbiochem (Darmstadt, Germany). All cell culture media and supplements, namely fetal bovine serum (FBS), glutamine and RPMI 1640, were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). Phenolic compounds such as catechin, epicatechin, kaempferol-3-gtlucoside, and procyanidins B1 and B2 were obtained from Extrasynthese (Genay, France). Chlorogenic acid and quercetin-3-glucoside were obtained from Sigma-Aldrich (Steinheimam Albuch, Germany).

2.2. APPLE CULTIVARS

A total of 26 apple cultivars examined in 2006 and 9 cultivars examined in 2007 were obtained from Estação Agrária de Viseu, Cooperativa Agrícola de Mangualde, Instituto de Formação e Educação Coop. (IFEC) and ex Direcção Regional de Agricultura do Entre-Douro e Minho (exDRAEDM). Moreover, some varieties were kindly provided by Engº. João Tomás Ferreira (Table 2.1 and 2.2). Apples were harvest successively as they were ripening from September to December of 2006 and 2007.

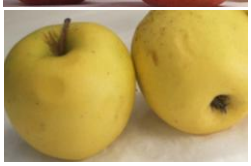
Table 2.1. Origin and harvest year of traditional Portuguese apples selected for this study

Cultivars		Origin	Harvest year
	*Bravo de Esmolfe*	Estação Agrária de Viseu; Cooperativa Agrícola de Mangualde	2006, 2007
	*Camoesa*	Cooperativa Agrícola de Mangualde	2006
	*Costa*	Cooperativa Agrícola de Mangualde	2006
	*Maçã Pedra*	Cooperativa Agrícola de Mangualde	2006
	*Maçã Verdeal*	ex Direcção Regional de Agricultura do Entre-Douro e Minho	2006
	*Malápio Fino*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Malápio IFEC*	Instituto de Formação e Educação Coop.	2006
	*Malápio do Jordão*	Cooperativa Agrícola de Mangualde	2006
	*Malápio da Ponte*	Estação Agrária de Viseu	2006

Table 2.1 (cont). Origin and harvest year of traditional Portuguese apples selected for this study

Cultivars		Origin	Harvest year
	*Malápio da Serra*	Estação Agrária de Viseu; Cooperativa Agrícola de Mangualde	2006, 2007
	*Pardo Lindo*	Estação Agrária de Viseu	2006
	*Pêro de Coura*	ex Direcção Regional de Agricultura do Entre-Douro e Minho	2006
	*Pêro da Lixa*	ex Direcção Regional de Agricultura do Entre-Douro e Minho	2006
	*Pêro Pipo*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Pêro Rei*	Estação Agrária de Viseu	2006
	*Pipo de Basto*	Cooperativa Agrícola de Mangualde	2006
	*Porta da Loja*	ex Direcção Regional de Agricultura do Entre-Douro e Minho	2006
	*Tromba de Boi*	Estação Agrária de Viseu	2006

Table 2.2. Origin and harvest year of exotic apples selected for this study

Cultivars		Origin	Harvest year
	*Fuji*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Gala Galaxy*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Golden*	Cooperativa Agrícola de Mangualde	2006, 2007
	*JF13*	Engº. João Tomás Ferreira	2006
	*JF29*	Engº. João Tomás Ferreira	2006
	*Reineta Parda*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Starking*	Cooperativa Agrícola de Mangualde	2006, 2007
	*Tarte*	Engº. João Tomás Ferreira	2006

2.3. EXTRACTION PROCEDURE

Apple extracts were prepared according to methods previously described (Dewanto et al., 2002; Eberhardt et al., 2000). The extractions were carried out with acetone/water mixtures as it allowed obtaining stable extracts due to the denaturation of polyphenol oxidase. In addition, a good extraction of polyphenols was assured using this solvent mixture. It has been reported that these compounds can be underestimated when alcoholic or hydroalcoholic extractions are used because most of them are not extracted (Amiot et al., 1992; Delage et al., 1991; Guoyot et al., 1998; Sanoner et al., 1999). In this work, the edible part of the fruits was extracted with 80% acetone (1:2, w/v) for 8 minutes in a blender. The homogenates were filtered and the solvent was evaporated in a rotary evaporator at 40ºC. The remaining extracts were diluted in distilled water to make a concentration of 2 g of fresh apple/mL. Finally, apple extracts were filtered through 0.22 μm filter (Sarstedt, Nümbrecht, Germany) before storage at -20ºC.

2.4. ANALYSES OF PHENOLICS

TOTAL PHENOLIC CONTENT

Total concentration of phenolic compounds present in apple extracts was determined according to the Folin Ciocalteau colorimetric method (Singleton and Rossi, 1965). Briefly, 20μL of the appropriate dilutions of extracts were added to 1480μL of distilled water and oxidized with Folin Ciocalteau reagent (100μL). The reaction was neutralized with 300μL of sodium carbonate and after 30 minutes at 40ºC of incubation the absorbance of samples was measured at 765 nm in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA). Gallic acid was used as standard, and the results were expressed as means of triplicates (mg of galic acid equivalents per 100g of edible fruits –mg GAE/100g).

HPLC ANALYSIS OF POLYPHENOLIC COMPOUNDS

HPLC analysis of phenolic compounds was carried out using a Surveyor equipment from Thermo Finnigan with a diode array detector (DAD) (Thermo Finnigan—Surveyor, San Jose, CA, USA) and an electrochemical detector (ED) (Dionex, ED40) (Bravo et al., 2006). The data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan—Surveyor, San Jose, CA, USA) and the software 4880 (Unicam) for the diode array and electrochemical detector, respectively. Identification of compounds was done by comparing retention time, spectra and spiking samples with known amounts of pure standards, whenever available. For quantification proposes standard solutions were prepared in methanol:water (50:50) solution and extracts were diluted 1:5 or 1:10 and analysed as triplicates. Since DAD is less selective than ED, the latest was used to quantify compounds that co-elute on the former detector, namely catechin, epicatechin, chlorogenic acid and procyanidins. Quercetin-3-glucoside was detected with both detection systems but quantified using data from DAD, due to the lower electrochemical signal. Moreover, using these chromatographic conditions, kaempferol-3-glucoside and quercetin-3-rahmonoside could not be separated and were quantified together as quercetin-3-glucoside equivalents. Phloridzin was not detected within this ED conditions and was only quantified from HPLC-DAD data.

2.5. ANTIOXIDANT ACTIVITY

OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

ORAC assay was used to evaluate the antioxidant capacity of the samples towards peroxyl radicals. The assay measures the ability of the antioxidant species present in the sample to inhibit the oxidation of FL catalysed by AAPH – generated peroxyl radicals ($ROO^{\bullet}$) and was carried using a modified method described by Ou et al. (2001). The composition of the reaction mixture was 6.3×10^{-8} M FL, 1.28×10^{-2} M AAPH (prepared in 75 mM PBS, pH 7.4) and the

appropriate diluted sample, making up a total volume of 1.8 mL. The reaction was started by the addition of AAPH to the mixture placed in a 10 mm wide fluorescence cuvette at 37ºC. Fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute at the emission wavelength of 515 nm and excitation wavelength of 493 nm in a fluorescence spectrophotometer with thermostatic bath, model Cary Eclipse (Varian LTD., Surrey, UK) for a period of 30 minutes. PBS was used as a blank and 1, 5, 12.5, 25 and 50 μM Trolox solutions were used as control standards. All samples, including the blank and the controls, were analysed in triplicate. Final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the FL decay curve. Data are expressed as micromoles of Trolox equivalents antioxidant capacity (TEAC) per 100g of apple and the experiment was performed in triplicate.

HYDROXYL RADICAL ADVERTING CAPACITY (HORAC)

HORAC assay was based on a previously reported method (Ou et al., 2002), modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA). This assay evaluates the hydroxyl radical prevention capacity of a sample using fluorescein (FL) as the probe. The hydroxyl radical was generated by a Co(II)-mediated Fenton like reaction and, similarly to ORAC assay, the fluorescence decay curve of FL was used to quantify the HORAC value. Briefly, 10 μL of appropriate dilutions of samples were added to 180μL of FL (4×10^{-3} μM) plus 5μL of CoF_2 (1.57 mg/mL). The reaction was started by the addition of 5μL H_2O_2 (1.1 M) to the mixture placed in a 96 well microplate at 37ºC. Fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute during 35 minutes. The FL800 microplate fluorescence reader was used with fluorescence filters for an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm, and the plate reader was controlled by software Gen5. Caffeic acid was used as a standard as it provides a wider linear range as compared to gallic

acid. Data was expressed as micromoles of caffeic acid equivalents (CAE) per 100 grams of fresh apples and all samples were analysed as triplicates.

HYDROXYL RADICAL SCAVENGING CAPACITY

Hydroxyl radical scavenging capacity of the apple extracts was evaluated by EPR (electron paramagnetic resonance) technique. This method involves the addition-type reaction of a short-lived radical with the paramagnetic compound DMPO to form a relatively long-lived free radical product, which can be detected by EPR. The intensity of the spectrum is related with the amount of free radicals trapped. Fenton reaction was used as a source of $HO^{\bullet}$ radicals. Briefly, hydroxyl radicals were generated by adding 100μL of 10 mM H_2O_2 to a solution containing 100μL of DMPO (48mM) and 100μL of $FeSO_4$ (2 mM in phosphate buffer solution, pH 7.4). Immediately, 100μL of apple extracts (2 g apple/mL) were added to the system and the spectrum of DMPO/$HO^{\bullet}$ was recorded 3 min later. Control experiments were carried out by adding 100 μL of distilled water. The scavenging capacities of apples extracts were determined by measuring the intensity of their spectrum and final results were expressed in percentage relative to the control. EPR measurements were conducted using an EPR Bruker EMX6/1 spectrometer (Bruker EMX6/1, Karlsruhe, Germany) and a flat cell assembly. Experiments were performed in duplicate, at room temperature and under atmospheric pressure.

INHIBITION OF AAPH INDUCED HUMAN LDL OXIDATION

Prior to oxidation, ethylenediamine tetraacetic acid (EDTA) in the LDL solution was removed by passage through a PD-10 desalting column (GE Healthcare, Buckinghamshire, UK), hydrated and eluted with PBS. Protein concentrations in LDL preparations were determined with Bradford reagent using BSA as a reference standard. For the oxidation assay, 0.1 mg/mL LDL and 500 μM of AAPH with or without apple extracts (0.1mg of apple/mL) were incubated in a

quartz cuvette at 37ºC. Light absorbance was read at 234 nm in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA) for a maximum period of 10 hours or until the LDL oxidation reached a plateau. Lag time was calculated by drawing two tangent lines: one through the initial, slowly rising curve which corresponded to the utilization of endogenous antioxidants in the LDL; and the other to a subsequent, rapidly rising curve which corresponded to the rapid LDL oxidation following the exhaustion of endogenous antioxidants. The intersection of two tangents was considered as the lag time, which is expressed in minutes. All samples were analysed in duplicates.

2.6. CELLULAR ANTIOXIDANT ACTIVITY

Human colon carcinoma Caco2 cells were purchased from (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and were routinely grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

To evaluate the intracellular antioxidant capacity of apple extracts, Caco2 cells were seeded at a density of 2×10^4 cells/well in 96 well plates and the medium was changed every 48 hours. The experiments were performed using completely differentiated cells (after reaching confluence- $\approx$72 hours) which are a good model of the intestinal barrier. This cell line, originally obtained from a human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005).

Intracellular antioxidant activity of the apple extracts was evaluated following the formation of reactive oxygen species (ROS) in cells after treatment with

two chemical stressors: t-BHP and H_2O_2. The formation of intracellular ROS was monitored using the fluorescent probe, DCFH-DA, as described previously (Wang and Joseph, 1999). Briefly, differentiated Caco2 cells were incubated for 24 hours with extracts equivalent to 100 mg/mL of apple. The cells were washed with PBS and incubated with 100 μM of DCFH-DA for 30 min. After removal of the DCFH-DA and further washing, the cells were incubated with 2 mM of t-BHP or 10 mM of H_2O_2 for 60min. The fluorescence of samples (F) was measured at 0 and 60 min in a microplate reader (FL600, Bio-Tek Instruments, Winooski, VT, USA). Intracellular antioxidant activity of apples was expressed as the percentage of inhibition of intracellular ROS caused by exposure to the oxidative stressors and was calculated as (1-(F60min-F0min)/F0min)x100 relatively to control (cells without apple extracts). Each experiment was repeated in quadruplicate.

2.7. ANTIPROLIFERATIVE ACTIVITY

Antiproliferative cell assays were done using human colon cancer cells HT29 (ATCC, Virginia, USA) and human gastric cancer cells MKN45 (kindly provided by Dr. Celso Reis -IPATIMUP, Oporto, Portugal). All cells lines were grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of glutamine. MKN45 medium was also supplemented with 50 μg/mL of gentamycin. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

Antiproliferative cell assays were performed as previously reported (Eberhardt et al., 2000). Briefly, cells were cultured in 96-well microplates at a density of 1×10^4 cells/well. After 24 hours incubation at 37ºC in 5% CO_2, the medium of each well was replaced by a medium containing the apple extracts (0-200 mg/mL). After 96 hours incubation, cell proliferation was determined using the colorimetric MTT assay. Results were expressed in terms of % of cellular viability relative to control (cells without apple extracts) and the amount of

sample necessary to decrease 50% of the cellular viability, ED_{50} (effective dose), was also calculated. The experiment was performed in triplicates.

2.8. STATISTICAL ANALYSYS

Data are reported as mean ± standard deviation (SD) of more than duplicates.

The NTSYS was used for Principal Component Analysis (PCA) of the promising apple varieties.

Comparisons between the apples harvested in 2007 with those obtained in 2006 were performed by Wilcoxon test. Statistical significance was established at $P<0.05$ or $P<0.01$.

3. RESULTS AND DISCUSSION

3.1. SELECTION OF PROMISING TRADITIONAL APPLES

The 26 varieties chosen for this study included 18 traditional apples and 8 exotic cultivars harvested in 2006. Firstly, apple samples were analysed for their total phenolic content using Folin Ciocalteau colorimetric assay. Results obtained are summarized in figure 2.1 and table 2.3.

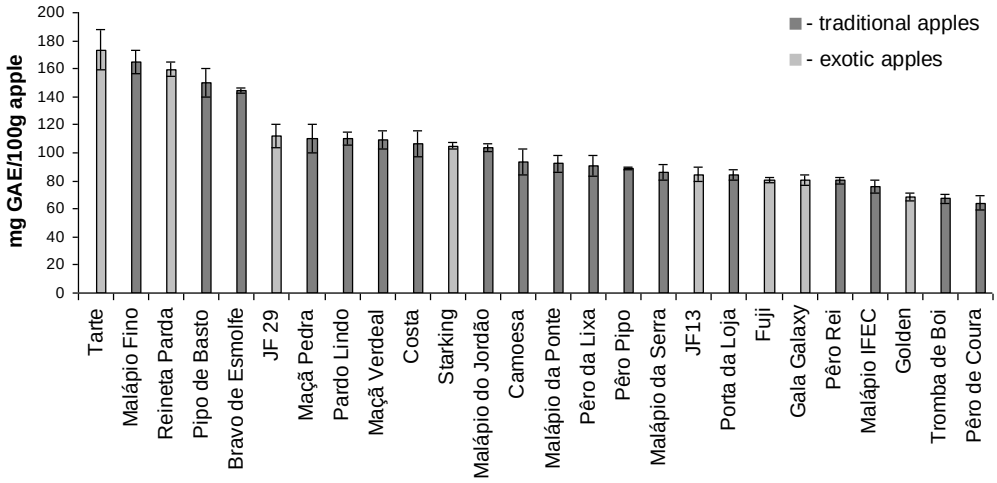


Figure 2.1. Total phenolic content of 26 apple varieties from Portugal

The average content of total polyphenols was 103.3 mg GAE/100g of fresh fruit, varying between 64.2 and 173.4 mg GAE/100g fw depending on apple variety. The present data are similar to those found in literature- 78.10-201.15 mg GAE/100g fw (Tsao et al., 2003) and 62.20-211.90 mg GAE/100g fw (Vrhovsek et al., 2004)- but it is known that these values can diverge in different years of production (Lata et al., 2005). The variability in total content of polyphenols manifested itself within the traditional apples and within the exotic varieties. The apples with higher levels of polyphenols were the exotics *Tarte* and *Reineta Parda* and the traditional cultivars *Malápio Fino*, *Pipo de Basto* and *Bravo de Esmolfe*, whereas the apples with lower polyphenol

content comprised *Golden*, *Tromba de Boi* and *Pêro de Coura* varieties (Figure 2.1). Moreover, the average of total content in polyphenols seems to be similar in traditional and exotic varieties (Table 2.3).

Table 2.3. Total phenolic content of 18 traditional and 8 exotic apple varieties

Traditional apples	mg GAE/100g
Bravo de Esmolfe	144.6
Camoesa	93.3
Costa	106.6
Maçã Pedra	110.4
Maçã Verdeal	109.0
Malápio Fino	164.7
Malápio IFEC	76.1
Malápio do Jordão	104.0
Malápio da Ponte	92.2
Malápio da Serra	86.0
Pardo Lindo	109.9
Pêro de Coura	64.2
Pêro da Lixa	90.7
Pêro Pipo	88.6
Pêro Rei	80.1
Pipo de Basto	150.2
Porta da Loja	84.4
Tromba de Boi	67.3
Average	**101.2**

Exotic apples	mg GAE/100g
Fuji	80.3
Gala Galaxy	80.3
Golden	68.9
JF13	84.6
JF 29	111.9
Reineta Parda	159.5
Starking	105.0
Tarte	173.4
Average	**108.0**

Taking into account these results, 17 varieties were chosen for the evaluation of their antioxidant potential. Economic factors and the agronomic potential of the fruit were also involved in this selection. The 17 apples included 9 regional varieties, namely:

- *Bravo de Esmolfe*, which is widely produced (annual production of 6000 ton) and very appreciated by consumer due to its unique flavour and aroma characteristics;

- *Camoesa*, *Malápio da Serra*, *Malápio Fino*, *Pêro Pipo*, *Pardo Lindo*, *Pipo de Basto* and *Porta da Loja*, that had higher commercial value in the 50's;

- *Malápio da Ponte*, identified by "Estação Agrária de Viseu" as derived from a plant with high agronomic potential.

The antioxidant activity of these apples was compared with eight exotic cultivars (*Fuji*, *Golden*, *Gala Galaxy*, *JF16*, *JF29*, *Starking*, *Reineta Parda* and *Tarte*), using three different and complementary in vitro assays: ORAC assay (scavenging of peroxyl radicals - $ROO^{\bullet}$), EPR assay (scavenging of hydroxyl radicals - $HO^{\bullet}$) and inhibition of human LDL oxidation.

Table 2.4. Antioxidant activity of 17 apple varieties

Traditional Apples	ORAC	EPR	LDL
Bravo de Esmolfe	1503	91	37
Camoesa	1232	90	23
Malápio Fino	2236	96	58
Malápio da Ponte	1111	64	42
Malápio da Serra	1389	65	28
Pardo Lindo	1739	98	21
Pêro Pipo	1277	100	28
Pipo de Basto	1732	58	42
Porta da Loja	1277	47	18
Average	**1500**	**79**	**33**

Exotic Apples	ORAC	EPR	LDL
Fuji	1065	65	23
Gala Galaxy	761	33	18
Golden	821	70	14
JF13	810	54	14
JF 29	1114	60	47
Reineta Parda	1533	55	32
Starking	1486	42	28
Tarte	1763	52	35
Average	**1169**	**54**	**26**

ORAC, Oxygen radical absorbance capacity (values are expressed in μmol TEAC/100 g apple); **EPR**, scavenging capacity of hydroxyl radicals measured by Electron Paramagnetic Resonance technique (results are presented in % of signal reduction induced by 0.5g apple/mL); **LDL**, inhibition of human Low Density Lipoprotein oxidation (values are expressed in % of lag phase retardation by the presence of 0.1 mg/mL of apple extract)

Results obtained for 17 apples are present in table 2.4 and it seems to diverge greatly between different varieties. The effect of apple extracts on antioxidant activity measured by ORAC assay ranged from 761 to 2236 μmol TEAC/100g fw; for EPR assay, they ranged from 33 to 100% of $HO^{\bullet}$ signal reduction; and for inhibition of LDL oxidation assay the retardation of lag phase varied between 14 and 58% (Figure 2.2).

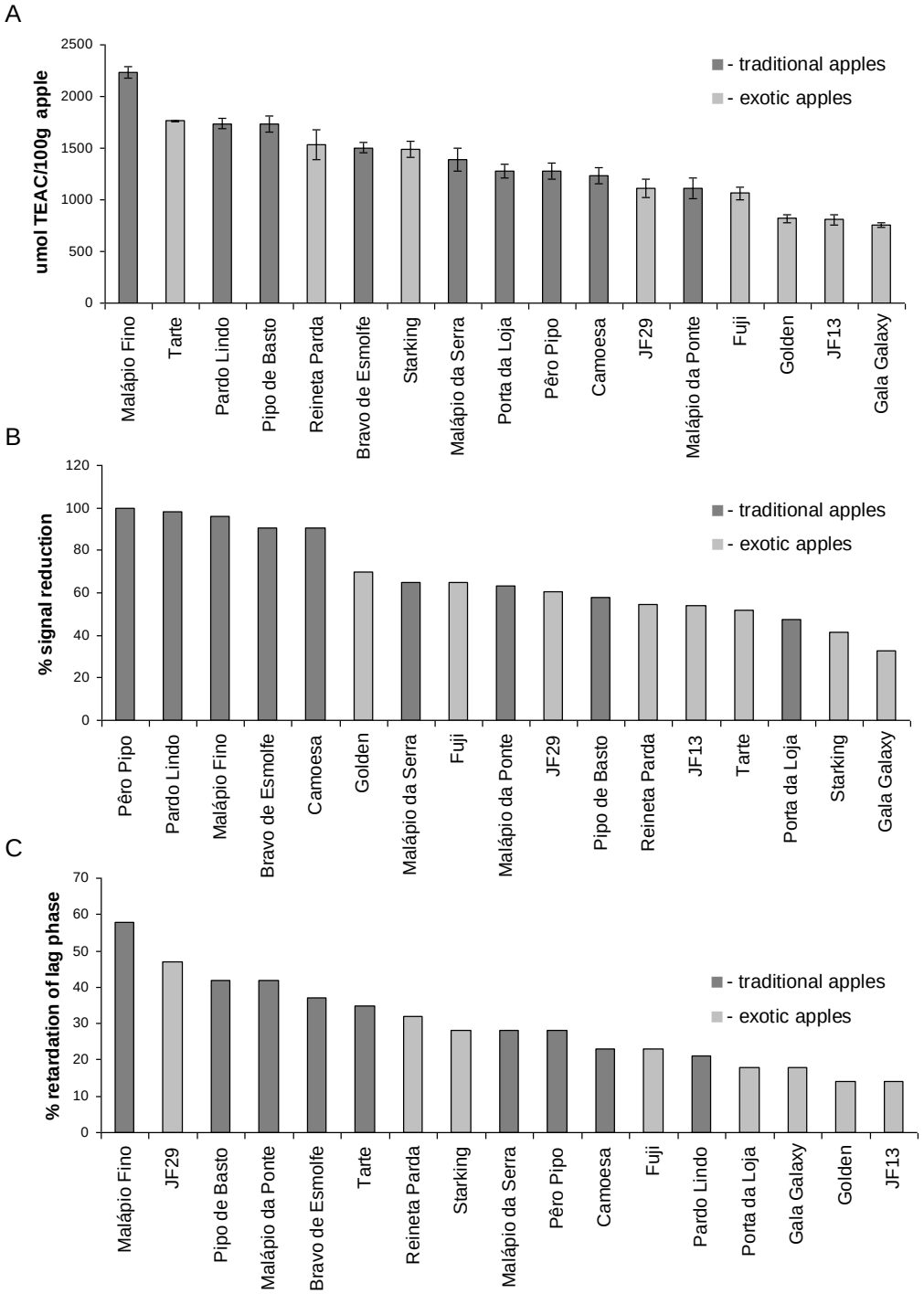


Figure 2.2. Antioxidant activity of traditional and exotic apple varieties: A- Scavenging of peroxyl radicals (ORAC assay); B- Scavenging of hydroxyl radicals measured by EPR technique (Figure A.1, Appendix A); C- Inhibition of AAPH induced LDL oxidation

It is important to highlight that, for all three assays, the average of antioxidant value was higher in traditional varieties (Table 2.4). *Malápio Fino* possessed the greatest activity in scavenging peroxyl radicals and in inhibiting human LDL oxidation and *Pêro Pipo* was the most efficient apple in reducing $HO^{\bullet}$ signal measured by EPR assay (Figure 2.2). On the other hand, the apples with lower antioxidant capacity comprised the exotics *Golden*, *Gala Galaxy* and *JF13*. These differences in antioxidant values of apples can be preliminary attributed to their different content in polyphenols.

The results obtained for ORAC assay were consistent with those reported by other authors. Lotito and Frei (2004) measured the antioxidant activity using ORAC assay in three apple cultivars, namely *Red Delicious*, *Granny Smith* and *Fuji*, and they found values of 1508, 785 and 830 μmol TEAC/100g, respectively. For the others assays, until now, no data is available for different apple varieties.

From all the results obtained in antioxidant assays, the apple cultivars were classified as those who present weak (+), medium (++), good (+++) and strong (++++) antioxidant activity (Table 2.5).

Table 2.5. Classification of apples according to their antioxidant activity, namely, scavenging capacity of peroxyl (ORAC) and hydroxyl radicals (EPR) and inhibitory effect on AAPH induced human LDL oxidation (LDL)

Traditional Apples	ORAC	EPR	LDL
Bravo de Esmolfe	+++	+++	+++
Camoesa	++	+++	++
Malápio Fino	++++	+++	++++
Malápio da Ponte	++	++	+++
Malápio da Serra	++	++	++
Pardo Lindo	+++	+++	++
Pêro Pipo	++	++++	++
Pipo de Basto	+++	++	+++
Porta da Loja	++	+	+

Exotic Apples	ORAC	EPR	LDL
Fuji	++	++	++
Gala Galaxy	+	+	+
Golden	+	++	+
JF13	+	++	+
JF 29	++	++	+++
Reineta Parda	+++	++	++
Starking	++	+	++
Tarte	+++	++	+++

+ weak; ++ medium; +++ good; ++++ strong

Results obtained are summarized in figure 2.3 and pointed out *Malápio Fino* as a powerful antioxidant apple followed by other four regional varieties, namely *Bravo de Esmolfe*, *Pardo Lindo*, *Pêro Pipo* and *Pipo de Basto*, and one exotic cultivar (*Tarte*). In contrast, the apple cultivars with lower antioxidant value were *Golden*, *Gala Galaxy*, *JF13* and *Porta da Loja* apples.

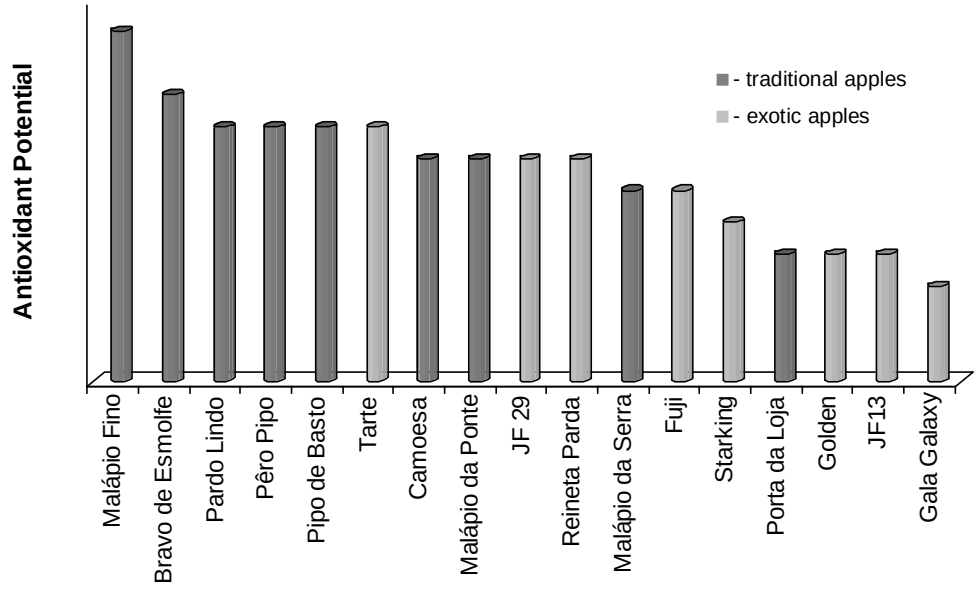


Figure 2.3. Antioxidant potential of 17 apple cultivars

From all the results obtained four traditional apples, namely *Bravo de Esmolfe*, *Malápio Fino*, *Pêro Pipo* and *Malápio da Serra*, were selected for further studies. Economic factors and the agronomic potential of the fruit were also involved in this selection. Moreover, five exotic apples which have economic significance in the current consumption of apples in Portugal, namely *Golden*, *Starking*, *Fuji*, *Gala Galaxy* and *Reineta Parda*, were chosen for comparison with traditional cultivars.

3.2. BIOACTIVITY EVALUATION OF PROMISING APPLE VARIETIES

In an effort to distinguish promising functional fruits, the next step of this work was to evaluate the bioactive effect of the nine apple cultivars.

On a first approach, the apple extracts were analyzed by HPLC in order to quantify their composition in bioactive compounds, namely polyphenols. Chromatograms obtained showed that apples had similar phenolic pattern but large variations in the content were detected between samples (Figures B1 and B2, Appendix B). The major phenolics identified were catechin, epicatechin, chlorogenic acid, procyanidin B1, procyanidin B2, phloridzin and the minor compounds were quercetin-3-glucoside, quercetin-4-glucoside, quercetin-3-rhamnoside and kaempferol-3-glucoside. Only quercetin-4-glucoside could not be quantified because it was below the quantification limit of the method. The remaining phenolics were quantified in apple extracts and results are presented in table 2.6.

Table 2.6. Phytochemical composition of traditional and exotic apples expressed as mg/100g of edible portion

Compounds	Traditional apples					Exotic apples					
	BE	**MF**	**MS**	**PP**	***Average***	**F**	**GG**	**G**	**RP**	**S**	***Average***
Cat	6.36	8.38	1.58	1.01	**4.33**	1.05	0.61	0.58	2.17	1.98	**1.28**
Ep	9.11	13.47	7.75	5.86	**9.05**	3.82	3.88	2.13	12.20	7.36	**5.88**
CAc	8.19	37.09	12.04	16.92	**18.56**	10.96	13.25	6.37	25.51	7.83	**12.78**
Ph	0.59	1.33	1.24	0.73	**0.97**	1.10	0.28	0.79	4.74	2.73	**1.93**
Q3g	0.11	0.39	0.05	0.13	**0.17**	0.16	0.07	0.06	0.10	0.09	**0.10**
K3g+Q3r	0.35	0.32	0.25	0.39	**0.33**	0.40	0.32	0.50	0.18	0.19	**0.32**
PB1	1.41	2.20	0.83	0.80	**1.31**	0.60	0.41	0.28	1.63	1.30	**0.85**
PB2	1.70	2.31	2.33	3.49	**2.46**	2.46	1.29	1.44	4.03	2.73	**2.39**

BE, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*; **Cat**, catechin; **Ep**, epicatechin; **Cac**, chlorogenic acid; **Ph**, phloridzin; **Q3g**, quercetin-3-glucoside; **K3g**, kaempferol-3-glucoside; **Q3r**, quercetin-3-rhamnoside; **PB1**, procyanidin B1; **PB2**, procyanidin B2

As shown in table 2.6, the traditional variety *Malápio Fino* had the highest contents of catechin, epicatechin, chlorogenic acid, quercetin-3-glucoside and procyanidin B1 whereas the exotic cultivar *Reineta Parda* contained the highest amounts of phlorizin and procyanidin B2. In spite of having the highest concentration of kaempferol-3-glucoside + quercetin-3-rhamnoside, *Golden* apple together with *Gala Galaxy* and *Fuji* had the lowest concentrations of polyphenols. Moreover, mean values for phenolic compounds in traditional varieties are higher or similar to exotic ones except for phloridzin, as mentioned previously. Phloridzin was detected in a much higher concentration in *Reineta Parda* and *Starking* apples and so the average content for the exotic varieties was increased. It is reasonable to conclude that in general the traditional varieties are a richer source of phenolic compounds than exotic varieties consumed worldwide.

The values reported in this study are slightly higher than those reported in previous works. In particular, Valles et al. (1994) found that *Starking* apple has 1.4mg/100g of phloridzin whereas *Reineta* has 4.5mg/100g of phloridzin and 1.5mg/100g of epicatechin. Moreover, Vrohovsek et al. (2004) reported that *Reineta* has 0.84mg/100g of catechin. These variations can be attributed to different years of harvest (climate conditions) and/or geographical location of fruits.

In summary, polyphenolic content varied with apple cultivar and thus it is expected that their biological effect could diverge substantially. The next step of this work was to evaluate and compare the antioxidant and antiproliferative effect of these nine apple cultivars using cell-based assays. The antioxidant effect was studied in differentiated Caco2 cells submitted to oxidative stress generated by t-BHP and H_2O_2. The antiproliferative activity of apples was tested in human colorectal (HT29) and gastric (MKN45) cancer cells.

Results obtained in all cell-based assays (Figure 2.4) showed that, the nine apple cultivars exhibited antioxidant protection at cellular level and inhibited

the proliferation of cancer cells. However, these activities diverged greatly within cultivars.

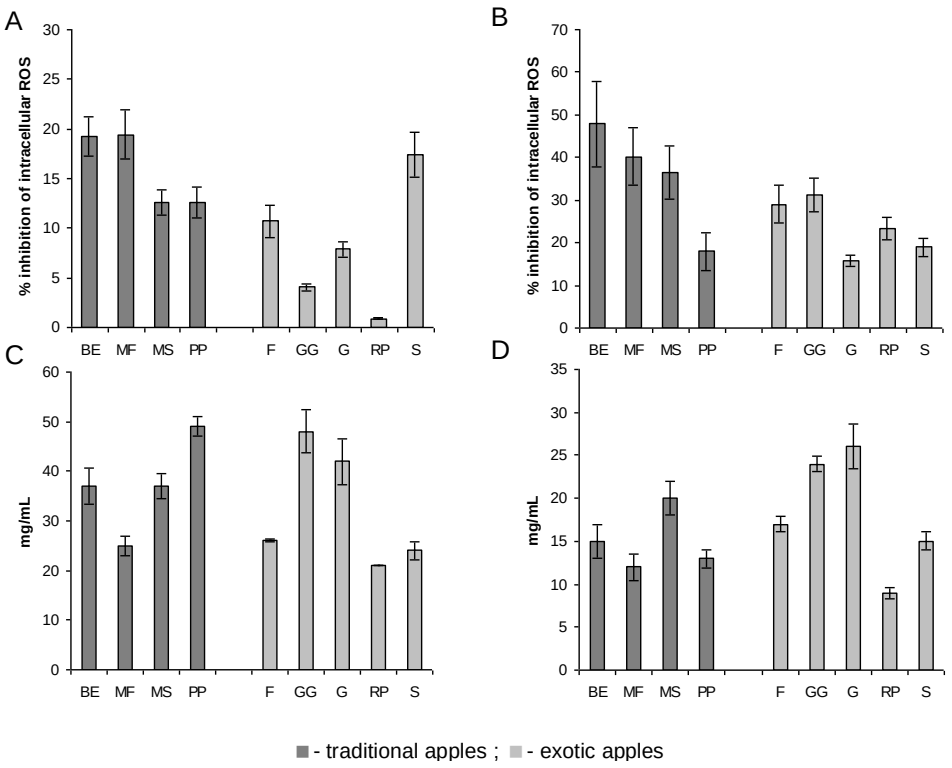


Figure 2.4. Bioactivity of nine apple varieties: A - Intracellular antioxidant activity against H_2O_2 induced oxidative stress in Caco2 cells; B- Intracellular antioxidant activity against t-BHP induced oxidative stress in Caco2 cells; C- ED_{50} values of HT29 cell proliferation; D- ED_{50} values of MKN45 proliferation. Numeric values are presented in table C.1 (Appendix C)
(**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

In order to distinguish the apples according to their biological activity, PCA was used in this study as it is a useful statistical tool to evaluate the pattern of correlation between variables and to detect groups among samples (Feliciano et al., 2009; Kallithraka et al., 2001a; Kallithraka et al., 2001b). Concerning this, 17 variables measured in nine apples were included in PCA evaluation (Table 2.7). In addition to the cellular antioxidant and antiproliferative assays,

antioxidant values of apples obtained using the three complementary *in vitro* chemical assays, namely ORAC, EPR and LDL (Table 2.4) were included in this analysis. HORAC assay was also performed in order to measure the efficacy of the apples to prevent hydroxyl radical formation. Finally, the phenolic composition was also included aiming to understand which compounds are responsible for the bioactivity of fruits.

Table 2.7. Numbers and abbreviations of 17 variables considered for PCA of apples

Number	Abbreviation	Variable designation
1	**TPC**	Total polyphenolic concentration
2	**Cat**	Catechin content
3	**CAc**	Chlorogenic acid content
4	**Ep**	Epicatechin content
5	**Ph**	Phloridzin content
6	**Q3g**	Quercetin-3-glucoside content
7	**K3g+Q3r**	Kaempferol-3-glucoside and quercetin-3-rhamnoside content
8	**PB1**	Procyanidin B1 content
9	**PB2**	Procyanidin B2 content
10	**ORAC**	$ROO^{\bullet}$ scavenging capacity measured by ORAC assay
11	**HORAC**	Prevention of $HO^{\bullet}$ formation capacity measured by HORAC assay
12	**EPR**	$HO^{\bullet}$ scavenging capacity measured by EPR assay
13	**LDL**	Inhibition of LDL oxidation
14	**HT29**	ED_{50} values towards HT29 proliferation
15	**MKN45**	ED_{50} values towards MKN45 proliferation
16	**t-BHP**	Intracellular antioxidant capacity towards t-BHP induced oxidative stress
17	**H_2O_2**	Intracellular antioxidant capacity towards H_2O_2 induced oxidative stress

In figure 2.5 is presented the projection of the samples (identified as letters) and variables (identified as numbers) in the bidimensional space defined by the first two principal components contributing to 77.92% of the total variance of the results. Two main groups were found: group A which is located in quadrants I and IV and group B in quadrant III.

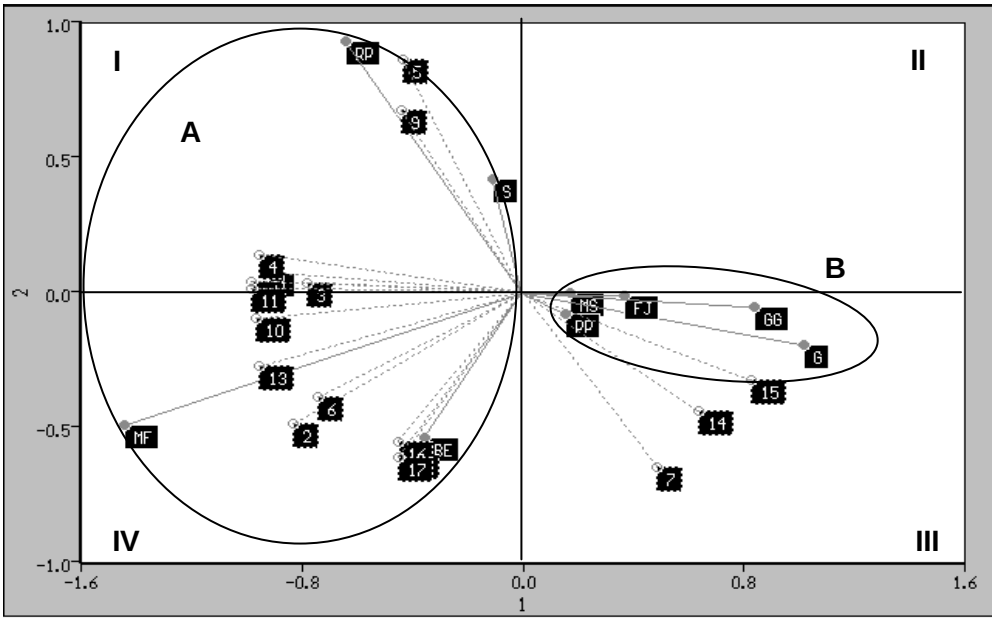


Figure 2.5. Distribution of four traditional apples and five exotic varieties along principal components 1 and 2, using 17 variables (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

Group A includes the apples with the highest polyphenolic content and also the best results achieved by ORAC and HORAC antioxidant assays, namely *Reineta Parda*, *Starking*, *Malápio Fino* and *Bravo de Esmolfe*. Within this group, Portuguese apple variety *Malápio Fino* was distinguished from the other apples due to its highest antioxidant effect for all *in vitro* chemical assays. This apple had the highest ORAC and HORAC values (Figures 2.2.A and 2.6) and was also the most effective in preventing human LDL oxidation (Figure 2.2.C). As shown in figure 2.7, *Malápio Fino* strongly retarded LDL oxidation when compared to the other three cultivars. The highest content in polyphenols as catechin, epicatechin, chlorogenic acid, quercetin-3-glucoside and procyanidin B1 contributed to discriminate this apple (Figure 2.5). These results suggest that these phenolic compounds could be responsible for the ORAC and HORAC values of apples and also for the capacity of preventing LDL oxidation. According to this, good correlations (>0.725) were obtained between the concentrations of catechin, epicatechin, quercetin-3-glucoside

and procyanidin B1 and the antioxidant activities measured by ORAC, HORAC and LDL assays (Table 2.8).

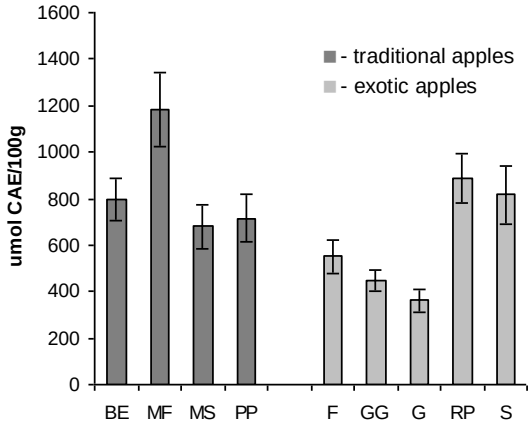


Figure 2.6. HORAC values of nine apple varieties (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

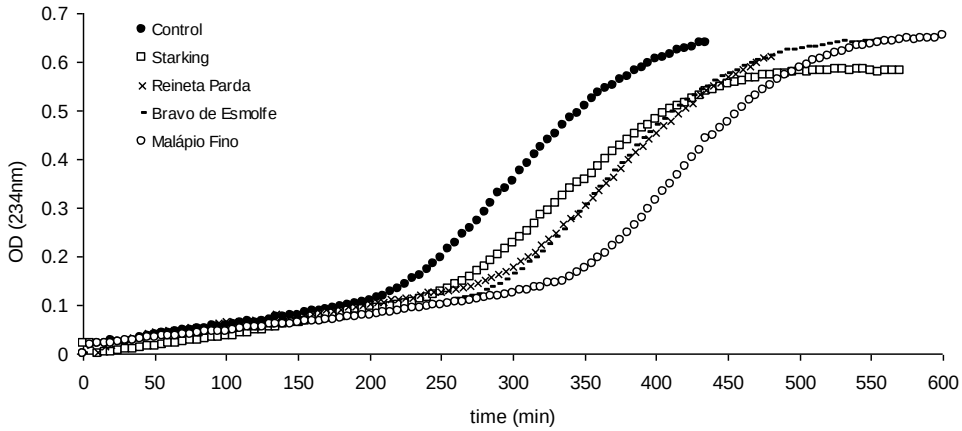


Figure 2.7. Kinetics of LDL oxidation by 500μM of AAPH as measured by conjugated dienes (OD_{234}) in control LDL and LDL in the presence of 0.1 mg/mL of apple extracts

These polyphenols were detected by HPLC with electrochemical detection (Figure B2, Appendix A) that was reported to be an important tool for detection of compounds that may contribute to higher ORAC values (Feliciano et al.,

2009). Our results also agree with previous work (Zessner et al., 2008) which reported good correlation between the peroxyl radical scavenging capacity and low molecular weight polyphenols (chlorogenic acid, catechin, epicatechin and quercetin glycosides) in apple juice extracts.

Table 2.8. Correlation values between phenolic content of apples and both antioxidant and antiproliferative tests

	***In vitro* antioxidant activity**				**Intracellular antiox. activity**		**Antiproliferative activity**	
	ORAC	**HORAC**	**EPR**	**LDL**	**t-BHP**	**H_2O_2**	**HT29**	**MKN45**
TPC	0.834	0.875	0.317	0.840	0.478	0.253	-0.588	-0.775
Cat	0.839	0.800	0.559	0.920	0.719	0.661	-0.367	-0.484
CAc	0.732	0.764	0.345	0.782	0.223	0.026	-0.381	-0.607
Ep	0.917	0.943	0.310	0.882	0.456	0.290	-0.596	-0.796
Ph	0.347	0.424	-0.322	0.167	-0.298	-0.339	-0.730	-0.611
Q3g	0.752	0.726	0.535	0.842	-0.342	0.480	-0.398	-0.473
K3g+Q3r	-0.459	-0.541	0.428	0.316	0.106	0.028	0.547	0.535
PB1	0.956	0.976	0.345	0.927	0.425	0.443	-0.656	-0.805
PB2	0.379	0.470	0.127	0.234	-0.362	-0.184	-0.430	-0.805

ORAC, $ROO^{\bullet}$ scavenging capacity measured by ORAC assay; **HORAC**, prevention of $HO^{\bullet}$ formation capacity measured by HORAC assay; **EPR**, $HO^{\bullet}$ scavenging capacity measured by Electron Paramagnetic Resonance technique; **LDL**, inhibition of human Low Density Lipoprotein oxidation; **t-BHP**, intracellular antioxidant capacity towards t-BHP induced oxidative stress; **H_2O_2,** intracellular antioxidant capacity towards H_2O_2 induced oxidative stress; **HT29**, antiproliferative effect (ED_{50} values) towards human colon cancer cells; **MKN45**, antiproliferative effect (ED_{50} values) towards human gastric cancer cells; **Cat**, catechin; **Ep**, epicatechin; **Cac**, chlorogenic acid; **Ph**, phloridzin; **Q3g**, quercetin-3-glucoside; **K3g**, kaempferol-3-glucoside; **Q3r**, quercetin-3-rhamnoside; **PB1**, procyanidin B1; **PB2**, procyanidin B2

Malápio Fino also had a good scavenging effect towards hydroxyl radicals as it strongly reduced the EPR spectra (Figure A.1, Appendix A). Another Portuguese variety, namely *Bravo de Esmolfe* apple, demonstrated the same effect. However, within group A, this apple stands out from the others for its higher intracellular antioxidant effect (Figure 2.5). *Bravo de Esmolfe* was the most active apple towards free radicals produced by t-BHP and H_2O_2 in Caco2 cell line, followed by *Malápio Fino* (Figures 2.4A, B). According to figure 2.5, catechin can contribute to this effect since it is present in higher

concentrations in these two traditional varieties; as reported previously, *Malápio Fino* and *Bravo de Esmolfe* had three to fourteen times more catechin than the other seven apple varieties (Table 2.6). For intracellular antioxidant activity towards t-BHP and H_2O_2 - induced oxidative stress, the best correlation was also obtained for catechin (Table 2.8). Furthermore, catechin has been reported to possess a high absorption and permeability in Caco2 cells (Deprez et al., 2001), a well-established model of human intestinal absorption. This might explain why this natural compound has a more efficient antioxidant activity.

Other apples included in group A comprise two exotic cultivars: *Starking* and *Reineta Parda*. These apples were distinguished from the others due to their highest concentration in phloridzin and procyanidin B2 and their powerful antiproliferative effect on both human colon and gastric cancer cells (lowest ED_{50} values) (Figure 2.5). Herein it is important to highlight that *Malápio Fino* also had a good antiproliferative effect against these human cancer cells (Figures 2.4C,D). As far as the other apple varieties are concerned, all induce similar results in the antiproliferative assays, affecting considerably the growth of human stomach cells only. The results obtained from the correlation analysis show that phloridzin and procyanidin B can contribute to antiproliferative activity of apples on human colon cancer cells as the best inverse relationship was obtained between ED_{50} values and their content (Table 2.8). For the inhibition of human gastric cells (MKN45) proliferation, the most significant correlations were obtained for total polyphenols, epicatechin, procyanidins B1 and procyanidin B2 contents. These apple compounds are also reported to have antiproliferative effect against several human cancer cells. Veeriah et al. (2006) reported antiproliferative activity of some apple phenolic compounds, such as phloridzin, quercetin, caffeic acid and chlorogenic acid against human colon HT29 cells. Zessner et al. (2008) showed that cytotoxic effect of apple juice extracts towards HCT119 colon cancer cells was correlated with the concentration of procyanidins. Moreover, Yoshizawa et al. (2005) found that catechin and epicatechin seemed partially

responsible for the antiproliferative activity of apples on human leukemic HL-60 cells.

Group B included the apple cultivars with lower polyphenolic content and bioactivity. Within this group, two subgroups were formed: one group constituted by *Golden* and *Gala Galaxy* and the other by *Fuji*, *Malápio da Serra* and *Pêro Pipo*. The first group included the cultivars that presented the lowest polyphenolic content and the lowest antioxidant and antiproliferative activities (highest ED_{50} values) (Figure 2.5). Fuji and the other two Portuguese varieties, *Malápio da Serra* and *Pêro Pipo*, were also confined in the quadrant IV but nearly to the centre of the bidimensional plot. These three apples had an intermediate value for antioxidant and antiproliferative activities. Although *Pêro Pipo* was the only apple that totally scavenged hydroxyl radicals (Figure A1, Appendix A), the variables used in this PCA did not discriminate this sample from the others.

In summary, the results obtained in this study pointed out traditional varieties *Malápio Fino* and *Bravo de Esmolfe* as powerful antioxidant apples and exotic *Reineta Parda* the best cultivar in promoting antiproliferative effect against human colon (HT29) and stomach (MKN45) cancer cells. Correlation studies demonstrated that catechin, epicatechin and procyanidin B1 are the major contributors to the antioxidant activity of apples whereas procyanidins (B1 and B2), phloridzin and epicatechin play an important role against human digestive cancer cell proliferation. However, it is important to highlight that apples contain a complex mixture of bioactive ingredients and thus the reported bioactivities are not only the sum of the contributions of different components but are also a result of synergies likely established among the bioactive compounds present.

3.3. TWO-YEARS COMPARISON OF APPLES

In order to study the effect of year cultivation on the health promoting activity of apples, the four promising traditional varieties, namely *Bravo de Esmolfe*, *Malápio Fino*, *Malápio da Serra* and *Pêro Pipo*, and five exotic cultivars (*Fuji*, *Gala Galaxy*, *Golden*, *Reineta Parda* and *Starking*) harvested in 2007 were analyzed for their polyphenolic content, antioxidant activity and antiproliferative effect. Results were then compared with those obtained in 2006 using Wilcoxon test.

Total polyphenolic content of 2007 apples varieties was measured by Folin Ciocalteau assay and the major phenolic compounds were quantified by HPLC-DAD-ED. Results are shown in figures 2.8 and 2.9. Concerning the total polyphenolic content, there were no differences between apples of two cropping years, for *Bravo de Esmolfe*, *Malápio Fino*, *Fuji*, *Gala Galaxy*, *Golden* and *Reineta Parda* (Figure 2.8).

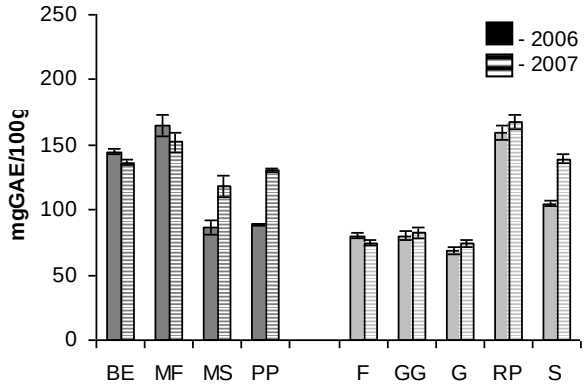


Figure 2.8. Comparison between total phenolic content of traditional and exotic apples harvested in 2006 and 2007. Statistically there was no significant difference between apples of these two crop years ($P > 0.05$). Numeric values of 2007 are presented in table D.1 (appendix D). (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

Pêro Pipo, *Malápio da Serra* and *Starking* apples showed increase of their polyphenolic content in 38%, 47% and 33%, respectively, in 2007. These variations were similar to those reported by (Stracke et al., 2009).

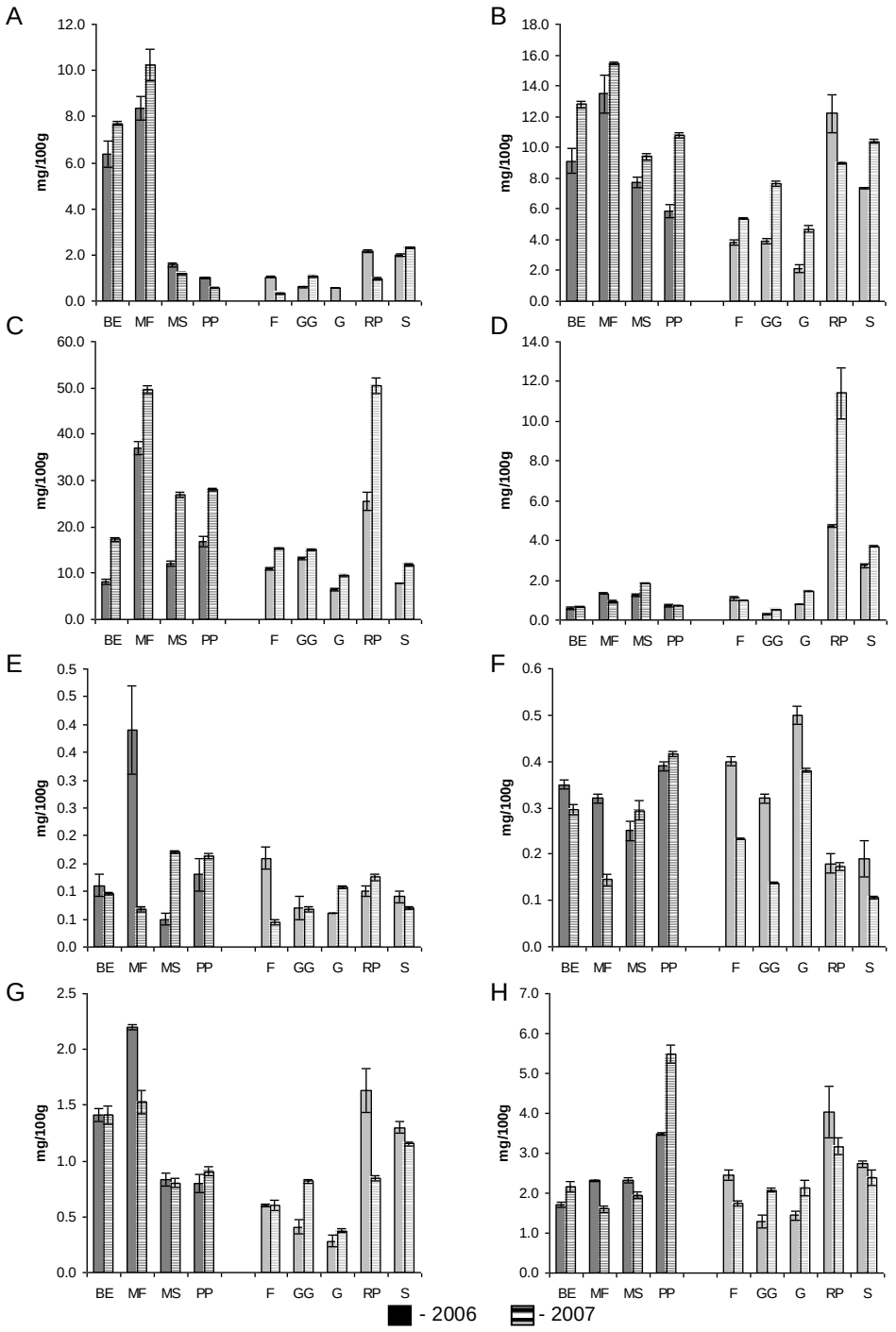


Figure 2.9. Comparison between phenolic content of traditional and exotic apple varieties harvested in 2006 and 2007. A- Catechin; B- Epicatechin; C- Chlorogenic acid; D- Phloridzin; E- Quercetin-3-glucoside; F- Kaempferol-3-glucoside + quercetin-3-rhamnoside; G- Procyanidin B1; H- Procyanidin B2. For catechin, epicatechin, quercetin-3- glucoside procyanidin B1 and B2 there was no significant difference between apples of two crop years ($P > 0.05$). Numeric values of 2007 were presented in table D.1 (Appendix D). (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

These authors evaluated the polyphenolic content of organically and conventionally produced apples of *Golden Delicious* variety over three years (2004-2006). In 2004 and 2005 they did not found any significant difference between the sums of phenolic compounds of apples whereas in 2006 highest concentrations were detected (33% and 44% of variation in relation to 2005, for organic and conventional apples respectively).

The phenolic composition of the different apples, harvested in 2006 and 2007 were compared and presented in figure 2.9. In some cases, there are significant differences between the two years relatively to the content of a specific phenolic compound.

Apples harvested in 2007 were also analysed for their antioxidant potential using EPR, ORAC and HORAC assays. Results were compared with 2006 and are shown in table 2.9.

Table 2.9. Antioxidant capacity of Portuguese apples (traditional and exotic varieties) of 2006 and 2007 crop years. Statistically, there was significant difference between apples of two crop years (ORAC: $P < 0.01$, HORAC and EPR: $P < 0.05$).

Apples	ORAC		HORAC		EPR	
	2006	2007	2006	2007	2006	2007
Traditional varieties						
Bravo de Esmolfe	1503 ± 48	2089 ± 60	796 ± 94	988 ± 82	90.7 ± 1.9	94.2 ± 1.9
Malápio Fino	2236 ± 56	2318 ± 44	1183 ± 157	1147 ± 15	96.2 ± 0.2	98.1 ± 0.2
Malápio da Serra	1389 ± 116	1703 ± 22	679 ± 93	640 ± 62	64.2 ± 0.1	97.8 ± 0.2
Pêro Pipo	1277 ± 79	1959 ± 56	715 ± 104	1029 ± 78	100 ± 0.9	94.7 ± 0.8
Average	**1601**	**2017**	**843**	**951**	**88**	**96**
Exotic varieties						
Fuji	1065 ±62	1210 ± 33	551 ± 71	621 ± 34	65.1 ± 0.3	95.8 ± 0.4
Gala Galaxy	761 ± 22	1311 ± 60	446 ± 45	917 ± 36	34.6 ± 4.7	51.7 ± 7.1
Golden	821 ± 39	1167 ± 67	360 ± 52	437 ± 21	69.8 ± 2.2	79.6 ± 2.5
Reineta Parda	1486 ± 75	2274 ± 36	886 ± 105	567 ± 24	54.7 ± 0.2	97.5 ± 0.3
Starking	1533 ± 141	1877 ± 225	816 ± 123	1053 ± 85	41.5 ± 0.3	95.3 ± 0.8
Average	**1133**	**1568**	**612**	**719**	**53**	**84**

ORAC, oxygen radical absorbance capacity (values are expressed in μmol TEAC/100 g apple); HORAC, hydroxyl radical adverting capacity (results are expressed in μmol CAE/100 g apple); **EPR**, scavenging capacity of hydroxyl radicals measured by Electron Paramagnetic Resonance technique (results are presented in % of signal reduction induced by 0.5g apple/mL)

For all three assays, there were significant differences between the antioxidant values of apples harvested in the two different years ($P<0.05$). In particular, for ORAC antioxidant assay, a significant increase was observed for 2007 apples ($P<0.01$). The highest variations (fold > 1.3) were found for *Bravo de Esmolfe*, *Pêro Pipo*, *Gala Galaxy*, *Golden*, *Reineta Parda* and *Starking* apples (Figure 2.10), in which concentrations of epicatechin and/or chlorogenic acid increased in 2007 (Figure 2.9B, C). Accordingly, these two phenolic compounds were already reported to have good correlations with ORAC values (Table 2.8).

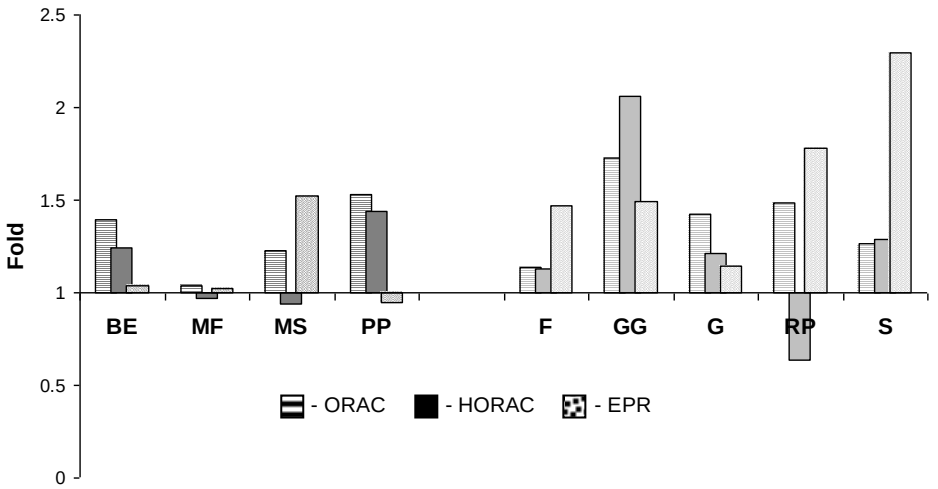


Figure 2.10. Variation of antioxidant capacity of apples between 2006 and 2007 crop years (values were obtained by the ratio between average values of 2007 divided by average value of 2006). (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

For HORAC assay*, Bravo de Esmolfe*, *Pêro Pipo*, *Gala Galaxy*, *Golden* and *Starking* apples harvested in 2007 also showed a higher increase on antioxidant value whereas *Reineta Parda* demonstrated a decrease in relation with 2006 crop year (Figure 2.10). As shown in figures 2.9B and 2.9E, these variations are mostly related with epicatechin and procyanidin B1 content, the main responsible compounds of HORAC values of apples (Table 2.8).

Finally, for EPR assay, the most relevant increases (fold>1.4) were obtained for *Malápio da Serra*, *Fuji*, *Gala Galaxy*, *Reineta Parda* and *Starking* (Figure 2.10). However, for this case it is still doubtful which phenolic compounds are the major responsible of scavenging capacity of hydroxyl radicals.

Overall, it seems that higher increases in antioxidant activities were obtained for exotic varieties (Figure 2.10). However, it is important to highlight that the average antioxidant value obtained for 2007 exotic apples was still lower than the average value of traditional varieties harvested in 2007 or even in 2006 (Table 2.9).

On contrary, *Malápio Fino* is the only cultivar that did not shown differences on antioxidant value in the two studied cropping years (Figure 2.10). Among all varieties analysed in 2007, this traditional variety was still the best antioxidant apple reaching the highest antioxidant values in the three chemical antioxidant assays (Table 2.9).

Stracke et al. (2009) also evaluated the antioxidant capacity of organically and conventionally produced *Golden Delicious* apples over 2004, 2005 and 2006, using three different assays, namely FRAP, ORAC and TEAC. For ORAC they found fold decreases of 0.8 between 2004 and 2005 for both production methods, and fold increases of 1.3 and 1.1 between 2005 and 2006 for organic and conventional apples, respectively. Additionally, the ORAC values of *Golden Delicious* apples reported by Stracke et al. (2009) were similar with those obtained in this study for *Golden* variety (5.0-11.4μmol TEAC/g fresh weigh).

Apples of 2007 were also analysed for their antioxidant capacity in *Caco2* cells. Results obtained demonstrated that there were no significant differences between the cellular antioxidant protection of apples harvested in two different years ($P>0.05$) (Figures 2.11A, B). This fact could be related with the low variation in the content of catechin (Figure 2.9A), compound that was previously pointed to be the major responsible of this effect (Table 2.8).

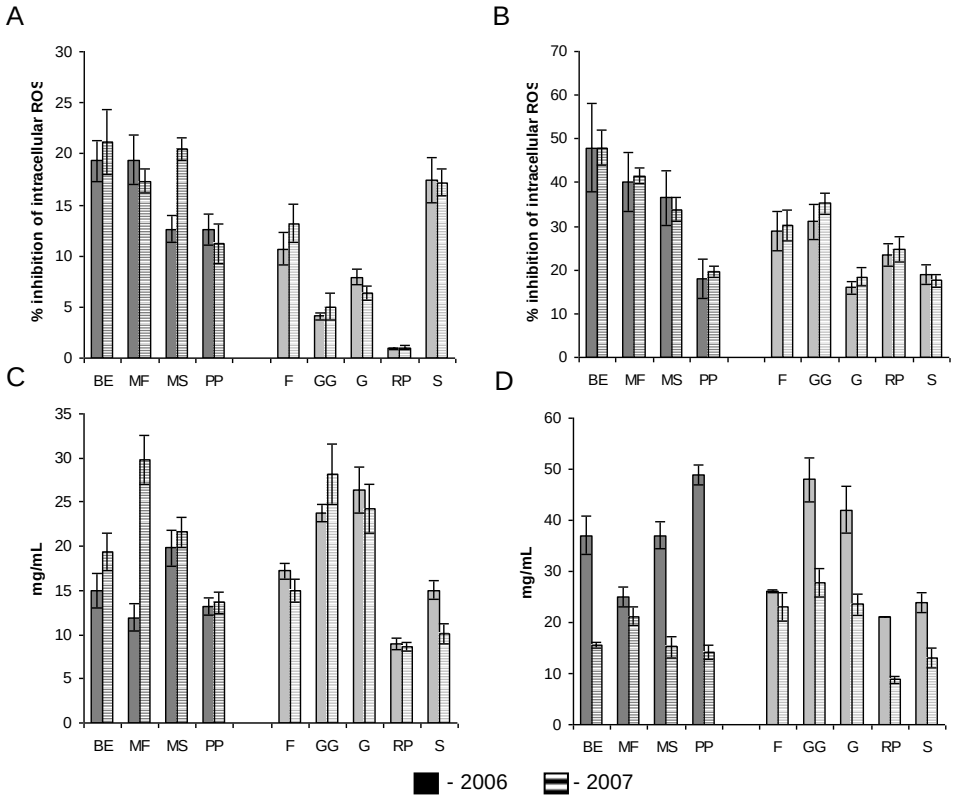


Figure 2.11. Comparison between bioactivity of traditional and exotic apples harvested in 2006 and 2007. A - Intracellular antioxidant activity against H_2O_2 induced oxidative stress in Caco2 cells; B- Intracellular antioxidant activity against t-BHP induced oxidative stress in Caco 2 cells; C- ED_{50} values of MKN45 cell proliferation; D- ED_{50} values of HT29 proliferation. Numeric values of 2007 were presented in table D.2 (Appendix D); (**BE**, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*)

For antiproliferative activity against human gastric cancer cells (MKN45), no significant variation was observed between the two crop years (Figure 2.11C) ($P<0.05$). But, in 2007, *Malápio Fino* apple showed a significant increase in ED_{50} value (decrease of antiproliferative effect), which could be associated with the decreased of procyanidin B1 content (Figures 2.11C, 2.9G). On contrary, significant increases were found for antiproliferative effect of 2007 apples against human colon cancer ($P<0.05$) (Figure 2.11D). The ED_{50} value of seven apple varieties (*Bravo de Esmolfe*, *Malápio da Serra, Pêro Pipo*,

Gala Galaxy, *Golden*, *Reineta Parda* and *Starking*) decreased in relation to 2006, probably due to the increase of phloridzin and epicatechin content (Figure 2.9 and Table 2.8). *Malápio Fino* and *Fuji* were the only ones that did not shown improvement of antiproliferative effect in HT29 cells.

Overall, the difference in phenolic composition and bioactivity of apples verified between the two crop years could be related with climate variations. In fact, less rainfall was observed in 2007 (525.1 L/m^2) than in 2006 (936.3 L/m^2) (http://www.meteo.pt), which may explain the higher content in specific polyphenols and antioxidant capacity of apple varieties from 2007. This effect was also verified by Stracke et al. (2009), when compared phenolic content of Golden Delicious apples produced in two different years.

Among all varieties of 2007, *Malápio Fino* and *Bravo de Esmolfe* were still the best antioxidant apples and *Reineta Parda* the most efficient in inhibiting the proliferation of human cancer cells (Figure 2.11). As verified in 2006, *Golden* and *Gala Galaxy* were the apples with lowest bioactivity in 2007. The weak antiproliferative effect achieved for *Malápio Fino* could be explained by its early maturation stage. In fact, this variety was harvested 2 months before optimal maturation stage which affect the concentration of phenolic compounds, in particular, those responsible for antiproliferative activity (Alonso-Salces et al., 2005).

4. CONCLUSIONS

The study reported herein describes the biological activity of traditional Portuguese apple cultivars and further comparison with exotic varieties.

Two traditional apples, namely *Bravo de Esmolfe* and *Malápio Fino*, which are only cultivated in specific regions of the North of Portugal, demonstrated to be rich sources of functional ingredients with powerful antioxidant activity and relevant antiproliferative effect against human cancer cells. The former being more adequate for human consumption as it was preferred by consumers (Feliciano et al., 2010) and the later as a promising raw material for the production of bioactive extracts.

The exotic *Reineta Parda* was the best cultivar in inhibiting human gastric and colon cancer cells proliferation. However, this apple presented a lower intracellular antioxidant effect.

The correlation between phenolic composition and bioactivity demonstrated that catechin, epicatechin and procyanidin B1 are the major contributors of the antioxidant activity of apples whereas procyanidins (B1 and B2), phloridzin and epicatechin play an important role against human cancer cell proliferation.

Finally, climatic conditions, in particular precipitation, influence phenolic content of apples and, consequently, their antioxidant and antiproliferative effect. Nevertheless, the major differences in biological activity occur within different varieties.

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PART II

CHERRIES

This chapter was based on the following manuscript:

Serra, A.T., Duarte, R.O., Bronze, M.R., Duarte, C.M.M. (2010). Identification of bioactive response in traditional cherries from Portugal, Food Chem, *accepted.*

ABSTRACT

In the recent years many studies on cherries revealed that they are rich sources of bioactive compounds with potential human health benefits.

In this work, it was evaluated the antioxidant activity and antiproliferative effect in human cancer cells of nine sweet cherries, including two traditional cultivars from Portugal (*Saco* and *Morangão*). Results obtained in biological assays together with the phenolic composition of cherries were submitted to principal component analysis which allowed samples to be grouped in terms of their bioactivity. *Saco* cherry and two exotic cultivars (*Ulster* and *Lapin*) showed the higher content in phenolic compounds, the best values of antioxidant activity and were the most effective in inhibiting the proliferation of human cancer cells derived from colon (HT29) and stomach (MKN45).

Correlation of the data obtained demonstrated that anthocyanins are the major contributors of the antioxidant capacity and antiproliferative effect of cherries. Additionally, hydroxycinnamic acids (neochlorogenic acid, chlorogenic acid and *p*-coumaroylquinic acid), flavan-3-ols (catechin and epicatechin) and flavonols (rutin and quercetin-3-glucoside) play also an important role in protection against oxidative stress.

TABLE OF CONTENTS

1. INTRODUCTION

Cherries are very attractive fruits to consumers due to their taste and colour attributes as well as for their wealth of nutrients. They are also a good source of natural antioxidant substances, namely polyphenols, which are reported to have many health benefits.

Portugal produces more than 15 thousands tons of cherries per year and it is in the northeast of the country, namely in the region of “Beira Interior”, where this fruit is more cultivated. In particular, “Cova da Beira” cherry has protected geographical indication (PGI) registration according to the EU regulations. This cherry is produced in the counties of Fundão, Covilhã and Belmonte, and covers both regional and non regional varieties. The fruits chosen for this study included two regional varieties, namely *Saco* and *Morangão*, which are old traditional Portuguese cultivars, and seven exotic cherries (*Summit*, *Maring*, *Van*, *Early Van Compact*, *Lapin*, *Ulster* and *Garnet*). The phenolic composition of some of these varieties has already been reported (Gonçalves et al., 2004a; Gonçalves et al., 2004b) however, there is few information concerning their antioxidant activity and no data about antiproliferative effect has been reported so far.

Within this context, the aim of this work was to evaluate and compare the bioactive effects of nine cherry cultivars, in an effort to distinguish promising functional fruits. For this purpose, cherries were characterized in terms of antioxidant potential and antiproliferative effect using human cancer cells. Polyphenolic content of fruits was also determined in order to understand which compounds are responsible for biological activities.

2. EXPERIMENTAL PROCEDURE

2.1. MATERIALS

Methanol (MetOH), 2',2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, cobalt floride tetrahydrate (CoF_2), bovine serum albumin (BSA), Bradford reagent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), hydrogen peroxide (H_2O_2) methylthiazolyldiphenyl - tetrazolium bromide (MTT), phosphate buffer solution (PBS) and t-butyl hydroperoxide (t-BHP) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Disodium fluorescein (FL) was obtained from TCI Europe (Antwerp, Belgium) and $FeSO_4$ was from Merck (Darmstadt, Germany). Human low density lipoprotein (LDL) was purchased from Calbiochem (Darmstadt, Germany). All cell culture media and supplements, namely fetal bovine serum (FBS), glutamine and RPMI 1640, were obtained from Invitrogen (Gibco; Invitrogen, Corporation, Paisley, UK).

Employed standars for HPLC analysis were catechin, epicatechin, chlorogenic acid, and *p*-coumaric acid all from Sigma-Aldrich (St. Louis, MO, USA), quercetin-3-O-glucopyranoside, rutin, cyanidin-3-glucoside and cyanidin-3-rutinoside all from Extrasynthèse (Genay, France).

2.2. CHERRY CULTIVARS

Sweet cherries from the cultivars *Saco*, *Morangão*, *Summit*, *Maring*, *Van*, *Early Van Compact*, *Lapin*, *Ulster* and *Garnet*, grown in "Cova da Beira" (Portugal), were randomly harvested by hand between May and June of 2008. Within 1 h of harvest all samples were packaged and frozen at -20 ºC for 4-10 weeks before analysis were carried out.

2.3. EXTRACTION PROCEDURE

Cherry extracts were prepared following the method described by Gonçalves et al. (2004a) with some modifications. Briefly, cherry seeds were carefully removed by hand and the edible part of the fruit was dehydrated in a freeze drier (Freeze Dryer Modulyo, Edwards, UK) at -40ºC, in the absence of light. Dried cherries (5 g) were milled and further extracted with 100mL of MetOH 60% at 2000rpm for 10 minutes. The homogenates were filtered under vacuum and the solvent was evaporated in a rotary evaporator at 40ºC. The remaining extracts were diluted in distilled water to make a final concentration of 0.2 g of dry cherry/mL. Finally, cherry extracts were filtered through 0.22 μm filter and storage at -20ºC until analysis.

2.4. ANALYSES OF PHENOLICS

TOTAL PHENOLIC CONTENT

The total concentration of phenolic compounds present in cherry extracts was determined according to the modified Folin Ciocalteau colorimetric method (Singleton and Rossi, 1965) as previously described (Chapter 2, Part I, section 2.4). Results were expressed in mg as gallic acid equivalents (GAE) per 100g of edible fruit (dry weight- dw).

TOTAL ANTHOCYANIN CONTENT

Total anthocyanins content was estimated by pH differential absorbance method as described by Wroslstad (Wroslstad, 2000). Briefly, absorbance of cherry extracts was measured at 510 and 700 nm in buffers at pH 1.0 (potassium chloride, 0.025M) and 4.5 (sodium acetate, 0.4M). Anthocyanin content was calculated using a molar extinction coefficient of 29.600 (cyanidin-3-glucoside) and absorbance of $A=[(A_{510}–A_{700})_{pH1.0}–(A_{510}–A_{700})_{pH4.5}]$. Results were expressed as mg cyanidin-3-glucoside (C3g) equivalents per 100g dw of edible fruit.

HPLC ANALYSIS OF PHENOLS AND ANTHOCYANINS

HPLC analysis of phenolic compounds was carried out using a Surveyor equipment from Thermo Finnigan with a diode array detector (Thermo Finnigan—Surveyor, San Jose, CA, USA) and an electrochemical detector (Dionex, ED40) (Bravo et al., 2006). The data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan—Surveyor, San Jose, CA, USA) and the software 4880 (Unicam) for the diode array and electrochemical detector, respectively. Identification of compounds was done by comparing retention time, spectra and spiking samples with known amounts of pure standards, whenever available. The quantities of the different phenolic compounds were assessed from peak areas and were calculated as equivalents of six representative standard compounds (from standard, linear regression curves of standards prepared in methanol and diluted in methanol:water 50:50 solution). Neochlorogenic acid, p-coumaroylquinic acid and catechin were quantified using HPLC-DAD detection at 280nm. Herein neochlorogenic acid was calculated as equivalents of chlorogenic acid whereas p-coumaroylquinic acid was determined as p-coumaric acid equivalents. Moreover, the flavonols quercetin-3-glucoside and rutin were quantified at 360nm, and calibration curves were obtained using standard compounds. Finally, for epicatechin and chlorogenic acid, electrochemical detection (ED) was used, due to co-elution.

For quantification of anthocyanins, the mobile phase used consisted of a gradient mixture of eluent A water:formic acid (90:10 v/v) and eluent B acetonitrile:water:formic acid (40:50:10 v/v/v). The following gradient of eluents was used: 0- 15min from 0 until 20% of eluent B; 10 min with 20% eluent B; 25–70 min, from 20 until 70% eluent B; 70–75 min, with 70% of eluent B; 75–85 min from 70 until 100% eluent B; 85–90 min, with 100% eluent B; 90-95 min from 100 to 0% of eluent B; and 95-100 min 100% of eluent A. The solvent flow rate was 0.7 mL/min. Acquisition range was set between 190 and 700 nm and chromatogram was monitored at 527 nm. Cyanidin glucosides were quantified using standard compounds whereas

pelargonidin-3-rutinoside, peonidin-3-glucoside and peonidin-3-rutinoside were calculated as cyanidin-3-glucoside equivalents.

Coefficients of variation on the HPLC quantifications were <5% and final concentrations were expressed as mg/100g dry weight.

2.5. ANTIOXIDANT ACTIVITY

OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

ORAC assay was used to evaluate the antioxidant capacity of the samples towards peroxyl radicals. The assay was carried out following the method of Huang et al. (2002) (described in Chapter 2, Part I, section 2.5), adapted for microplate fluorescent reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). This assay measured the ability of the antioxidant species presented in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH. Results were expressed as micromoles of trolox equivalents antioxidant capacity per gram of dry weight (μmol TEAC/g dw).

HYDROXYL RADICAL ADVERTING CAPACITY (HORAC)

The HORAC assay was adapted from Ou et al (2002) and modified for the FL800 microplate fluorescence reader as described previously (Chapter 2, Part I, section 2.5). Data were expressed as micromoles of caffeic acid equivalents per gram of dry cherries (μmol CAE/g dw).

HYDROXYL RADICAL SCAVENGING CAPACITY

The ability of the extracts to scavenge hydroxyl radicals generated by hydrogen peroxide (Fenton reaction) was evaluated by electron paramagnetic resonance (EPR) using DMPO as the spin trapping agent, as described

previously (Chapter 2, Part I, section 2.5). Briefly, hydroxyl radicals were generated by adding 100 μL of 10 mM H_2O_2 to a solution containing 100 μL of DMPO (48mM) and 100 μL of $FeSO_4$ (2 mM in phosphate buffer solution, pH 7.4). Immediately, 100 μL of cherry extracts (200 mg dw/mL) were added to the system and the spectrum of DMPO/HO• was recorded 3 min later. Control experiments were carried out by adding 100 μL of distilled water. The scavenging capacities of cherry extracts were determined by measuring the intensity of their spectrum and final results were expressed in percentage relative to the control. All EPR measurements were conducted using a Bruker EMX6/1 spectrometer (Bruker Instrumments, Billerica, MA) and a flat cell assembly. Experiments were performed in duplicate, at room temperature under atmospheric pressure.

INHIBITION OF AAPH INDUCED HUMAN LDL OXIDATION

The ability of cherry extracts to inhibit human LDL oxidation was evaluated by conjugated diene formation as described previously (Chapter 2, Part I, section 2.5). In this assay, human LDL (0.1mg/mL) was oxidized by peroxyl radicals generated by AAPH (500 μM) and conjugated dienes formation was monitored continuously at 234nm using a Genesys10uv spectrometer (Thermo Spectronic, New York, USA). The protective effect of cherry extracts (0.25 μg dw/mL) was achieved by lag phase retardation of human LDL oxidation. Final results were expressed in terms of percentage of lag time retardation relative to the control (without extract).

2.6. CELLULAR ANTIOXIDANT ACTIVITY

Human colon carcinoma Caco2 cells were purchased from DSMZ (Germany), and were routinely grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

To evaluate the cellular antioxidant capacity of cherry extracts, Caco2 cells were seeded at a density of 2×10^4 cells/well in 96 well plates and the medium was changed every 48 hours. The experiments were performed using completely differentiated cells (after reaching confluence- ≈72 hours). Cellular antioxidant activity of the apple extracts was evaluated following the formation of reactive oxygen species (ROS) in Caco2 cells after treatment with two chemical stressors: t-BHP and H_2O_2. The formation of intracellular ROS was monitored using the fluorescent probe, DCFH-DA, as described previously (Chapter 2, Part I, section 2.6). Briefly, for the pre-incubation assay differentiated Caco2 cells were washed with PBS and incubated for 4 hours with cherry extracts (10 mg dw/mL). Then cells were washed again with PBS and incubated with 100 μM of DCFH-DA for 30 min. After removal of the DCFH-DA and further washing, 2 mM of t-BHP or 10 mM of H_2O_2 were added to the cells for 60 min. For the co-incubation assay, cherry extracts were added to Caco2 cells at the same time of chemical stressors. For both assays, fluorescence (F) was measured for each sample at 0 and 60 min in a fluorescence microplate reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). Cellular antioxidant activity of cherry extracts was expressed as the percentage of inhibition of intracellular ROS caused by exposure to the oxidative stressors and was calculated as $(1-(F_{60min}-F_{0min})/F_{0min}) \times 100$ against a control (cells without cherry extracts).

2.7. ANTIPROLIFERATIVE ACTIVITY

Antiproliferative cell assays were performed in human colon cancer cells HT29 (ATCC, USA) and human gastric cancer cells MKN45 (kindly provided by Dr. Celso Reis -IPATIMUP, Portugal). All cells lines were grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

Antiproliferative cell assays were carried out as reported previously (Chapter 2, Part I, section 2.7). Briefly, cells were cultured in 96-well microplates at a density of 1×10^4 cells/well. After 24 hours incubation at 37ºC in 5% CO_2, the medium of each well was replaced by medium containing the cherry extracts (0-20 mg dw/mL). After 96 hours of incubation, cell proliferation was determined using the colorimetric MTT assay. Results were expressed in terms of % of cellular viability relative to control (cells without cherry extracts) and the amount of sample necessary to decrease 50% of the cellular viability, ED_{50} (effective dose), was also calculated. The experiments were performed in triplicates.

2.8. DATA TREATMENT

The NTSYS was used for principal component analysis (PCA).

3. RESULTS AND DISCUSSION

The biological activities of two traditional cherry varieties and seven exotic cultivars were evaluated using both cell-free and cell-based assays .

In a first approach, the nine cherry cultivars were characterized in terms of bioactive compounds, including polyphenols and anthocyanins, using HPLC. Electrochemical detection was used not only to quantify compounds that co-elute, but also to identify potential antioxidant ingredients as it detects electrochemically active substances (Feliciano et al., 2009). HPLC chromatograms of fruit extracts showed that cherries contained similar phenolic profile (Figure B.2, Appendix B). However, some variations in their content were detected between samples (Table 2.10) which means that cherries could exhibit different biological activities.

Table 2.10. Phytochemical composition of traditional and exotic cherries*

Cherry cultivars	TPC	TAC	TED	Flavan-3-ols		Hydroxycinnamates			Flavonols		Anthocyanins			
				Cat	Ep	NcAc	pCqAc	CAc	Rut	Q3g	C3g	C3r	Pl3r +Pn3g	Pn3r
Traditional varieties														
Morangão	555.3	5.6	81.2	3.8	2.4	74.3	5.6	4.6	14.9	0.8	<1.0	<1.0	<1.0	<1.0
Saco	1308.8	296.2	227.2	7.5	9.4	123.2	15.2	8.7	26.6	5.3	55.6	282.0	5.3	3.5
Exotic varieties														
EVCompact	956.1	223.5	98.3	5.8	1.8	55.8	3.7	3.0	31.7	3.3	26.4	206.1	3.6	2.0
Garnet	652.7	69.1	82.4	2.7	0.3	26.0	2.3	2.9	18.7	1.3	3.7	68.8	<1.0	<1.0
Lapin	1308.8	371.5	156.3	8.6	10.6	85.5	6.1	8.9	23.4	3.9	70.3	161.7	4.5	5.4
Maring	833.0	151.0	98.8	8.3	1.5	65.7	3.7	4.6	32.9	2.1	34.7	141.9	<1.0	<1.0
Summit	440.1	28.8	29.2	3.8	0.1	20.8	17.6	1.9	7.8	0.6	2.9	36.0	<1.0	<1.0
Ulster	1187.1	292.0	116.2	6.8	3.0	54.4	32.8	6.7	28.0	2.3	28.2	302.6	1.8	1.6
Van	998.9	251.0	114.0	7.4	2.6	61.9	4.6	5.3	34.2	3.5	70.1	252.9	1.8	2.5

*Results are expressed in mg/100g of dry weight except for TED which corresponds to total area of chromatogram obtained by electrochemical detection. Coefficients of variation on the HPLC quantifications were <5%

TPC, total polyphenolic content (expressed as mg of gallic acid equivalents/100g dw); **TAC**, total anthocyanin content (expressed as mg of cyanidin-3-glucoside equivalents/100g dw); **TED**, total area of chromatogram obtained with electrochemical detection; **Cat**, catechin; **Ep**, epicatechin; **NcAc**, neochlorogenic acid; **pCqAc**, p-Coumaroylquinic acid; **CAc**, chlorogenic acid; **Rut**, rutin; **Q3g**, quercetin-3-glucoside; **C3g**, cyanidin-3-glucoside; **C3r**, cyanidin-3-rutinoside; **Pl3r**, pelargonidin -3-rutinoside; **Pn3g**, peonidin-3-glucoside; **Pn3r**, peonidin-3-rutinoside

To evaluate and compare the bioactivity of cherries, fruit extracts were analysed for their antioxidant and antiproliferative effect. Antioxidant activity

was determined using four different and complementary *in vitro* chemical assays (ORAC, EPR, HORAC and LDL) and at a cellular level, using a Caco2 model submitted to oxidative stress induced by t-BHP or H_2O_2. Antiproliferative effect was performed in human colon (HT29) and gastric (MKN45) cancer cells.

Results obtained in all assays showed that the nine cherry varieties exhibited distinct antioxidant and antiproliferative activities (Table 2.11).

Table 2.11. Antioxidant and antiproliferative activities of cherry varieties

Cherry cultivars	*In vitro* antioxidant activity				Cellular antioxidant activity		Antiproliferative activity	
	ORAC	HORAC	EPR	LDL	t-BHP	H_2O_2	HT29	MKN45
Traditional varieties								
Morangão	65.5±8.0	44.3±5.2	32.1±3.1	4.2	15.1±1.5 **co** 16.0±1.1 **pre**	15.0±1.9 **co** 14.7±2.9 **pre**	13.8±1.8	16.3±1.1
Saco	172.0±7.3	132.4±5.5	41.7±2.0	6.5	23.9±3.0 **co** 15.4±1.8 **pre**	33.7±6.1 **co** 16.6±3.3 **pre**	5.4±0.4	6.5±0.6
Exotic varieties								
EVCompact	120.3±8.4	64.3±2.4	30.4±2.2	5.6	21.0±3.0 **co** 13.6±2.4 **pre**	19.2±1.7 **co** 13.4±1.7 **pre**	6.2±1.1	9.8±2.8
Garnet	65.7±4.6	49.1±6.1	41.5±3.6	3.7	13.2±1.3 **co** 14.6±2.3 **pre**	12.0±2.0 **co** 11.6±2.4 **pre**	6.9±0.2	13.8±2.5
Lapin	177.1±6.7	93.3±5.7	50.4±0.2	8.2	29.8±3.6 **co** 19.0±2.2 **pre**	31.3±4.6 **co** 15.8±3.5 **pre**	5.9±0.5	10.5±0.7
Maring	104.0±5.6	65.0±6.2	32.1±6.2	5.2	10.7±2.0 **co** 13.5±1.8 **pre**	20.1±1.6 **co** 19.7±3.2 **pre**	8.4±0.6	11.0±0.1
Summit	50.4±6.9	24.1±2.2	56.5±1.8	2.5	3.3±0.7 **co** 7.0±1.5 **pre**	6.6±1.0 **co** 8.4±2.7 **pre**	9.4±1.1	15.9±1.9
Ulster	155.6±5.4	102.6±8.1	68.4±3.6	5.8	19.3±1.0 **co** 15.1±2.2 **pre**	32.6±5.0 **co** 20.3±1.5 **pre**	4.3±0.1	5.6±1.2
Van	122.3±7.1	66.6±0.7	42.8 ± 7.6	5.6	21.6±4.1 **co** 14.6±4.0 **pre**	26.3±4.0 **co** 18.9±1.8 **pre**	5.0 ± 0.3	6.2 ± 1.6

ORAC, $ROO^{\bullet}$ scavenging capacity of cherry measured by ORAC assay (results are expressed as μmol of trolox equivalents /g dw); **HORAC**, Prevention of $HO^{\bullet}$ formation capacity measured by HORAC assay Hydroxyl Radical Adverting Capacity assay (results are expressed as μmol of caffeic acid equivalents /g dw); **EPR**, Scavenging capacity of cherry extracts against hydroxyl radicals, measured by EPR technique (% of signal reduction induced by 50mg dw/mL of cherry extract); **LDL**, inhibition of human LDL oxidation (lag time retardation induced by 0.25μg dw/mL of cherry extracts); **t-BHP**, cellular antioxidant capacity of cherry extracts (10mg dw/mL) towards t-BHP induced oxidative stress in Caco2 cells (co- and pre-incubation assay); **H_2O_2**, cellular antioxidant capacity of cherry extracts (10mg dw/mL) towards H_2O_2 induced oxidative stress in Caco2 cells (co- and pre-incubation assay; **HT29**, antiproliferative effect of cherry extracts against human colon cancer cells (ED_{50} values expressed as mg/mL); **MKN45**, antiproliferative effect of cherry extracts against human gastric cancer cells (ED_{50} values expressed as mg/mL)

Due to the large amount of data obtained, principal component analysis (PCA) was applied in order to distinguish cherry varieties according to their biological activity. This analysis has been successfully used in the treatment of data obtained from the study of different apple varieties (Serra et al., 2010) and also in wine studies (Feliciano et al., 2009; Kallithraka et al., 2001).

Table 2.12. Numbers and abbreviations of 24 variables considered for PCA of cherries

Number	Abbreviation	Variable designation
1	**TPC**	Total polyphenolic concentration
2	**TAC**	Total anthocyanin concentration
3	**Cat**	Catechin content
4	**Ep**	Epicatechin content
5	**NcAc**	Neochlorogenic acid content
6	**pCqAc**	p- Coumaroylquinic acid content
7	**CAc**	Chlorogenic acid content
8	**Rut**	Rutin content
9	**Q3g**	Quercetin-3-glucoside content
10	**C3g**	Cyanidin-3-glucoside content
11	**C3r**	Cyanidin-3-rutinoside content
12	**Pl3r + Pn3g**	Pelargonidin -3-rutinoside + Peonidin-3-glucoside content
13	**Pn3r**	Peonidin-3-rutinoside
14	**TED**	Total electrochemical area
15	**ORAC**	$ROO^{\bullet}$ scavenging capacity measured by ORAC assay
16	**HORAC**	Prevention of $HO^{\bullet}$ formation capacity measured by HORAC assay
17	**EPR**	$HO^{\bullet}$ scavenging capacity measured by EPR assay
18	**LDL**	Inhibition of LDL oxidation
19	**H_2O_2pre**	Cellular antiox. capacity towards H_2O_2 induced oxidative stress (pre-incubation)
20	**H_2O_2co**	Cellular antiox. capacity towards H_2O_2 induced oxidative stress (co-incubation)
21	**t-BHPpre**	Cellular antiox. capacity towards t-BHP induced oxidative stress (pre-incubation)
22	**t-BHPco**	Cellular antiox. capacity towards t-BHP induced oxidative stress (co-incubation)
23	**HT29**	ED_{50} values towards HT29 proliferation
24	**MKN45**	ED_{50} values towards MKN45 proliferation

In this study, 24 variables were measured in nine cherries and data were analysed by PCA (Table 2.12). The cumulative percentage of the total variance explained by the first two components was 78.4%. A bidimensional plot was designed (Figure 2.12) and the distribution of cherry cultivars along PC1 and PC2 shows that samples could be divided in four main groups: group A, which is located in quadrants I and IV and includes *Summit* (Sm), *Garnet*

(Gn) and traditional *Morangão* (Mo) cherry varieties; group B, positioned nearly to the centre of the bidimensional plot, and comprises *Maring* (Ma), *Early Van Compact* (EVC) and *Van* (Van) cherries; group C located in quadrant II, which includes *Lapin* (Lp) and the other traditional cherry variety (*Saco*- Sc); and finally, group D, located in quadrant III, that includes only one variety (*Ulster*- Ul).

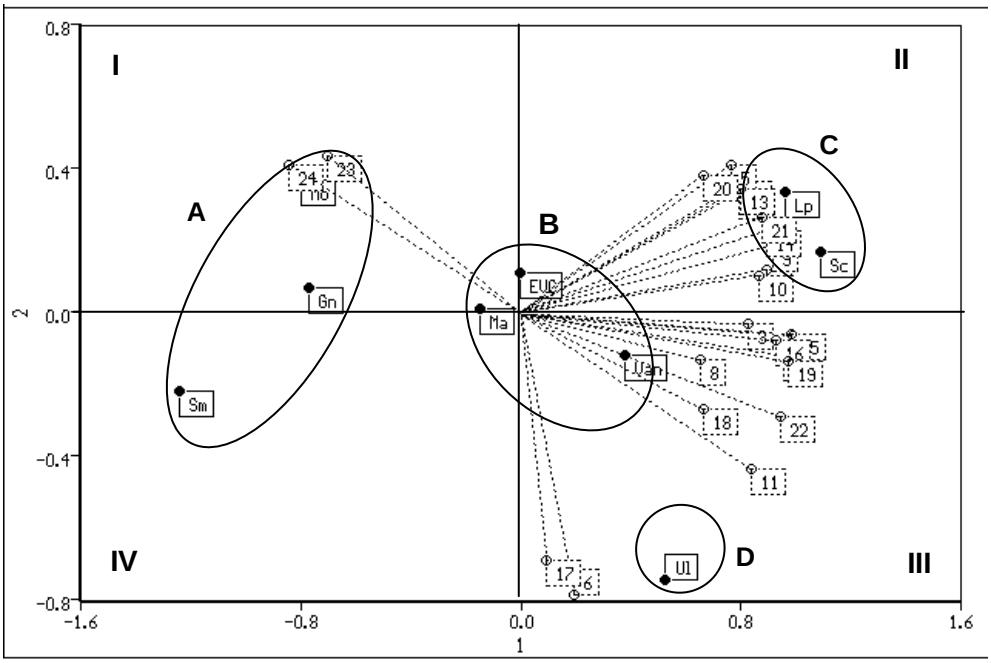

Figure 2.12. Distribution of nine Portuguese cherry varieties along principal component 1 and 2, using 24 variables (**Mo**, *Morangão*; **Sm**, *Summit*; **Gn**, *Garnet*; **Ma**, *Maring*; **EVC**, *Early Van Compact*; **Ul**, *Ulster*; **Lp**, *Lapin*; **Sc**, *Saco*)

In order to understand which variables accounted more for the distribution obtained, they were added to the bidimensional plot (Figure 2.12). Groups C and D include the cherries with the highest concentration in phenolic compounds and the best results of antioxidant activity. In particular, *Saco* and *Lapin* varieties (Group C) exhibited the highest ORAC and HORAC values and were the most effective in inhibiting human LDL oxidation (Table 2.11). Moreover, these two varieties distinguished from the others by their higher content in total polyphenols, total antocyaninis and, in particular, catechin,

epicatechin, neochlorogenic acid, chlorogenic acid, quercetin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-rutinoside + peonidin-3-glucoside and peonidin-3-rutinoside. The results obtained suggest that these compounds could be the major responsible for the HORAC and ORAC values obtained and also for the capacity of preventing LDL oxidation. In fact, high correlations (r> 0.83) were obtained between the total concentration of polyphenols and anthocyanins and the antioxidant activities measured by ORAC, HORAC and LDL assays (Table 2.14). Among all compounds, the best coefficients of correlation were achieved between chlorogenic acid and quercetin-3-glucoside with ORAC (r=0.888; 0.878,respectively), HORAC (r= 0.883; 0.846, respectively) and LDL (r= 0.864; 0.821, respectively) assays. Peonidin-3-rutinoside also exhibited an important effect on the prevention capacity of cherries to inhibit human LDL oxidation (r= 0.870).

Table 2.13. Correlation coefficients between phenolic content of cherries and both antioxidant and antiproliferative assays

	ORAC	HORAC	EPR	LDL	H_2O_2pre	H_2O_2co	BHPpre	BHPco	HT29	MKN45
TPC	0.994	0.931	0.154	0.923	0.630	0.965	0.652	0.872	-0.752	-0.847
TAC	0.972	0.832	0.192	0.916	0.583	0.915	0.573	0.850	-0.808	-0.841
TED	0.856	0.940	-0.071	0.787	0.498	0.856	0.644	0.783	-0.509	-0.674
Cat	0.823	0.680	-0.027	0.831	0.753	0.802	0.442	0.608	-0.528	-0.711
Ep	0.825	0.783	0.100	0.844	0.298	0.766	0.655	0.801	-0.344	-0.421
NAc	0.721	0.794	-0.211	0.724	0.506	0.744	0.620	0.689	-0.149	-0.472
CAc	0.888	0.883	0.233	0.864	0.604	0.913	0.735	0.797	-0.422	-0.607
pCqAc	0.283	0.370	0.851	-0.014	0.195	0.337	-0.205	-0.063	-0.294	-0.360
Rut	0.604	0.524	-0.339	0.605	0.784	0.634	0.419	0.530	-0.614	-0.799
Q3g	0.878	0.846	-0.162	0.821	0.439	0.820	0.512	0.820	-0.680	-0.770
C3g	0.818	0.661	-0.029	0.842	0.589	0.800	0.522	0.769	-0.640	-0.737
C3r	0.829	0.813	0.191	0.639	0.674	0.850	0.316	0.619	-0.859	-0.990
Pl3r + Pn3g	0.806	0.771	-0.092	0.766	0.131	0.680	0.465	0.784	-0.493	-0.507
Pn3r	0.805	0.640	0.092	0.870	0.210	0.699	0.613	0.848	-0.476	-0.433

ORAC, ROO• scavenging capacity; HORAC, prevention of HO• formation capacity; EPR, HO• scavenging capacity; LDL, inhibition of human LDL oxidation; **H_2O_2pre,** cellular antiox. capacity towards H_2O_2 induced oxidative stress (pre-incubation assay); **H_2O_2co,** cellular antiox. capacity towards H_2O_2 induced oxidative stress (co-incubation assay); **tBHPpre,** cellular antiox. capacity towards tBHP induced oxidative stress (pre-incubation assay); **tBHPco,** cellular antiox. capacity towards tBHP induced oxidative stress (co-incubation assay); **HT29**, antiproliferative activity (ED_{50} values) aganist human colon cancer cells; **MKN45**, antiproliferative activity (ED_{50} values) aganist human gastric cancer cells; **TPC**, total polyphenolic content; **TAC**, total anthocyanin content; **TED**, total area of chromatogram obtained with electrochemical detection; **Cat**, catechin; **Ep**, epicatechin; **NcAc**, neochlorogenic acid; **pCqAc**, p-coumaroylquinic acid; **CAc**, chlorogenic acid; **Rut**, rutin; **Q3g**, quercetin-3-glucoside; **C3g**, cyanidin-3-glucoside; **C3r**, cyanidin-3-rutinoside; **Pl3r**, pelargonidin -3-rutinoside; **Pn3g**, peonidin-3-glucoside; **Pn3r**, peonidin-3-rutinoside

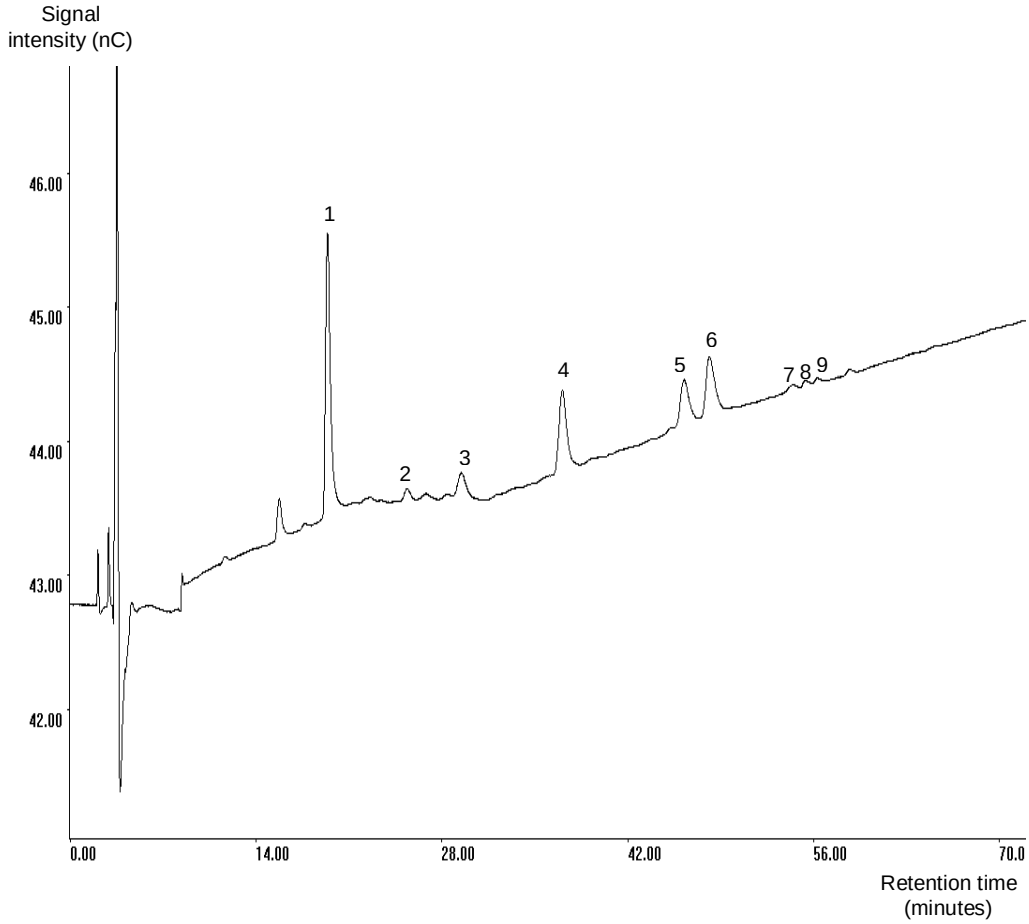

Figure 2.13. Chromatographic profile of *Saco* variety extract using ED (Legend: **1-** neochlorogenic acid; **2-** catechin; **3-** chlorogenic acid; **4-** epicatechin; **5-** cyanidin-3-glucoside; **6-** cyanidin-3-rutinoside; **7-** peonidin-3-rutinoside; **8-** rutin; **9-** quercetin-3-glucoside)

It is important to highlight that all these compounds were detected by HPLC with the electrochemical detection mode (Figure 2.13) which is an important tool to detect substances with antioxidant activity. Previous studies with apples and wines reported good correlations between total area of electrochemical chromatogram (TED) and ORAC values of samples (Feliciano et al., 2007; Feliciano et al., 2009). In this work, cherries of Group C contained the highest TED values (Table 2.11) and high coefficients of correlation were obtained between ORAC (r= 0.856), HORAC (r= 0.940) and LDL (r= 0.787) antioxidant assays (Table 2.13). Moreover phenolic

compounds identified by HPLC-ED were already reported by other authors to be active antioxidative substances in cherries. For example, Piccolella et al. (2008) studied the antioxidant properties of methanolic extracts from sour cherries and found that flavonoids (including catechin, epicatechin, quercetins and anthocyanins) and quinic acid derivatives (chlorogenic acid, neochlorogenic acid) are the most active substances in scavenging free radicals ($O_2^{\bullet-}$, NO and $ABTS^{+}$). Cyanidin and its derivatives are also recognized to be significant contributors for the antioxidant activity measured by ABTS assay, in tart cherry products (Kyrakosyan et al., 2009). For ORAC assay, Blando et al. (2004) showed that the antioxidant values of cherries are related with their total anthocyanin content whereas for HORAC assay, to date, no study has been published for cherry samples. In our previous study with apple cultivars (Serra et al., 2010), good correlations ($r > 0.725$) were obtained between antioxidant values of ORAC and HORAC with catechin, epicatechin and chlorogenic acid, suggesting that these compounds are the major contributors of antioxidant capacity in several fruit matrices.

Regarding LDL assay, Gonçalves et al. (2004b) reported that the antioxidant activity of Portuguese cherries on human LDL tend to correlate with catechin concentrations. In contrary to our results, these authors found a negative correlation with cyanidin-3-rutinoside levels and pointed out *p*-coumaroylquinic acid as the major contributor of antioxidant activity of cherries. This difference can be due to the use of different inductors on the oxidation assay; in this work we used an azo compound (AAPH) which generates peroxyl radicals by thermal decomposition, while Gonçalves and co-authors used transition metal ions derived from $CuSO_4$.

Among all compounds studied, *p*-coumaroylquinic acid was identified to be the only responsible for hydroxyl radical scavenging capacity (EPR assay) in cherry cultivars (Table 2.14). The highest content of this phenolic acid was detected in *Ulster*, the cherry variety most effective in reducing EPR spectrum (Table 2.11 and Figure A.2, Appendix A). It is important to note that these two

variables together with the powerful antiproliferative effect on both human colon and gastric cancer cells (lowest ED_{50} values) contributed to distinguish this cherry from the others - Group D (Figure 2.12). As shown in table 2.11, *Ulster* exhibited the highest antiproliferative effect in both cancer cells followed by *Saco*, *Van* and *Lapin*, which were the varieties with higher content in anthocyanins. According to this, the results obtained from the correlation analysis show that total anthocyanins and, in particular, cyanidins glycosides, are the major responsible for the inhibition capacity of human colon and gastric cancer cells growth (Table 2.13). These substances are widely reported in literature to have anticarcinogenic activities in cell culture models and also in animal model tumor systems (Wang and Stoner, 2008).

Cherries of groups C and D distinguish from the others due to their higher antioxidant activities in cellular assays (Figure 2.12). For co-incubation methodology (when cells were co-incubated with cherry extracts plus chemical stressors) total phenolic content and, in particular, total anthocyanins concentration of cherries seemed to be the major responsible for the cellular antioxidant protection ($r > 0.85$, Table 2.13). In fact, the cherries with higher total anthocyanin concentration (>200mg/100g dw, Table 2.10), namely *Saco*, *Lapin*, *Ulster*, *Early Van Compact* and *Van*, were the ones that exhibited the highest antioxidant effect in the two co-incubation assays (Figure 2.14). Moreover, when comparing the results obtained in co- and pre-incubation tests, these five cultivars showed a significant decrease on antioxidant protection in the later assay. This difference could be explained by the presence of anthocyanins, which are compounds with lower absorption in Caco2 cell monolayers but powerful scavengers of extracellular free radicals (Yi et al., 2006). The other four cultivars showed similar results in both assays, which means that phenolic compounds with higher permeability in Caco2 could be the main responsible agents for the cellular antioxidant response. Accordingly, for pre-incubation assays, the best correlations were obtained between tBHPpre and chlorogenic acid concentration ($r = 0.735$) and for H_2O_2pre and catechin ($r = 0.753$) and rutin ($r = 0.784$) content (Table 2.13).

These compounds were already reported to have good absorption (Deprez et al., 2001; Konishi and Kobayashi, 2004) and also antioxidant protection in Caco2 cells submitted to oxidative stress (Aherne and O'Brien, 1999). Additionally, results obtained with different apple varieties show that catechin was the major contributor for the intracellular antioxidant protection in the same cellular model (Serra et al., 2010).

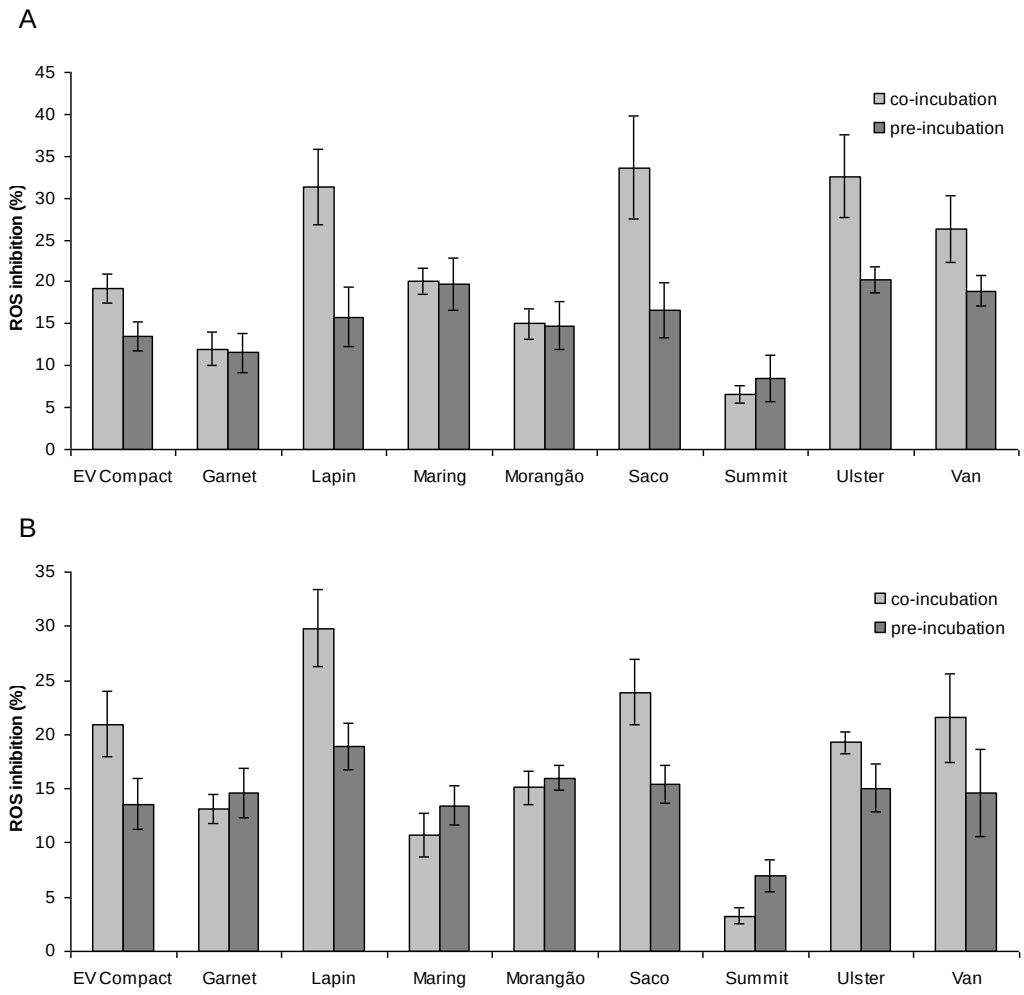

Figure 2.14. Effects of cherry extracts (10 mg dw/mL) on (A) H_2O_2 and (B) t-BHP induced ROS in Caco2 cells, measured by 2',7'-dichlorofluorescin oxidation. Comparison between co- and pre-incubation assays

Samples in Group B are located in the center of the bidimensional plot and include the cherry varieties with moderate polyphenolic content, antioxidant and antiproliferative activities (*Early Van Compact*, *Maring* and *Van*) (Figure 2.12). Among the three, *Van* variety contained higher concentration in phenolic compounds and bioactivity, which are the variables that account more for PC1.

Finally, Group A includes *Summit*, *Garnet* and traditional *Morangão* cherries which are the varieties with lower phenolic content, antioxidant activity and antiproliferative effect.

4. CONCLUSIONS

The study reported herein describes the antioxidant activity and antiproliferative effect towards human cancer cells of two traditional Portuguese cherry cultivars (*Saco* and *Morangão*) and further comparison with seven exotic varieties. The results obtained pointed out *Saco* variety as a rich source of bioactive ingredients with higher antioxidant capacity and antiproliferative effect in human cancer cells. These traditional cherry together with exotic cultivars *Lapin* and *Ulster* can be considered as promising functional foods for human health applications.

Correlation of the data obtained showed that anthocyanins are the major contributors of the antioxidant capacity and antiproliferative effect of cherries. Additionally, hydroxycinnamic acids (neochlorogenic acid, chlorogenic acid and *p*-coumaroylquinic acid), flavan-3-ols (catechin and epicatechin) and flavonols (rutin and quercetin-3-glucoside) play also an important role in protection against oxidative stress.

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6. ACKNOWLEDGMENTS

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CHAPTER 3

DEVELOPMENT OF FUNCTIONAL INGREDIENTS

In this chapter, the aim was to isolate functional ingredients from traditional fruits, in particular from the *Saco* cherry culls (fruit that is not suitable to sell for eating due to appearance defects, including small size), and *Malápio Fino* apple which was the variety with the highest bioactive effect but was the least preferred by consumers.

All the processes used were based on the application of clean technologies including the utilization of biocompatible solvents (eg. water and ethanol) high pressure technology and adsorption process.

PART I

ISOLATION OF AN ANTICANCER EXTRACT FROM CHERRY (*SACO* VARIETY), USING HIGH PRESSURE EXTRACTION

This chapter was based on the following manuscripts:

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ABSTRACT

In the recent years many studies on cherries revealed that they are rich sources of bioactive compounds with potential anticancer effect.

In this work, high pressure extractions were performed on a traditional sweet cherry variety from Portugal (*Saco*) in order to recover natural ingredients with anticancer activity. The methodology employed comprised a first step with supercritical CO_2 followed by a second step where different mixtures of CO_2 and ethanol (10-100% v/v) were tested. All extractions were performed at 50ºC and 25 MPa during 1 plus 1.5 hours and the resulting extracts were then characterized in terms of global yield, phenolic content, antioxidant activity and antiproliferative effect in human colon cancer cells.

The product obtained with CO_2:EtOH (90:10 v/v) extraction exhibited the highest antioxidant activity and was the most effective in inhibiting the growth of human colon cancer cells. When comparing with doxorubicin, this extract induced cell cycle arrest in a different cell cycle check point suggesting that this product can be used in combination with the drug in cancer chemotherapy.

In order to obtain extracts with enhanced antiproliferative activity, the extraction process was further explored. By using highly pure CO_2 and EtOH 96% the inhibition of cancer cell growth was significantly enhanced by 16 fold. In addition, the incorporation of a conventional extraction step with MetOH or EtOH:H_2O (50:50 v/v) prior to multi-step high pressure process allowed to obtain cherry extracts more concentrated in antiproliferative compounds. Perillyl alcohol present in cherry extracts was pointed to be one of the major responsible for anticancer properties.

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1. INTRODUCTION

Cancer is one of the most leading causes of death worldwide. According to a recent report by the World Health Organization (WHO), the disease accounted for 7.9 million deaths (or around 13% of all deaths worldwide) in 2007 and the projection is that this number will increase up to 18 million in 2020 (WHO, 2007). In particular, colorectal cancer is the third most common form of cancer in men (after prostate and lung) and the second in women (after breast). Diet and lifestyle are pointed to be major risk factors for developing this type of cancer (Parkin et al., 1995).

Epidemiological data suggest that ingestion of bioactive compounds from fruits and vegetables, such as polyphenols and terpenes, may contribute to reduce the incidence of cancer in humans (Kris-Etherton et al., 2002). The mechanisms by which these compounds inhibit tumorgenesis include inhibition of tumour cell mediated protease activity (Yang et al., 2001) attenuation of tumour angiogenesis (Kandaswami et al., 2005), induction of cell cycle arrest (Jayaprakasha et al., 2009; Mu et al., 2007; Wiseman et al., 2007) and promotion of apoptosis (Ho et al., 2009; Mertens-Talcott and Percival, 2005). In addition, it has been reported that combining natural compounds with chemotherapeutic agents is a promising strategy to enhance the inhibition of tumour survival (Chang et al., 2001; Chinery et al., 1997).

Sweet cherry (*Prunus avium*) is a much appreciated fruit by consumers due to its taste, colour and sweetness. Moreover, cherries constitute a rich source of bioactive compounds, namely flavonoids (anthocyanins, flavan-3-ols and flavonols) (Gao and Mazza, 1995; Gonçalves et al., 2004) that are already reported to exhibit anticancer properties (Beattie et al., 2005; Kang et al., 2003; Lila, 2004). In particular, cherry contains perillyl alcohol (Kris-Etherton et al., 2002), a hydroxylated monocyclic monoterpene, which has been shown to have a potential use as an anticancer drug by stimulating different types of tumour to apoptosis (Burke et al., 2002; Elegbede et al., 2003), inhibiting their proliferation (Bardon et al., 2002; Bardon et al., 1998; Yuri et al., 2004) or

overcoming their resistance to chemo-/radiotherapy (Rajesh et al., 2003; Samaila et al., 2004). Perillyl alcohol has also been investigated by the National Cancer Institute (NCI) in NCI-sponsored phase I, II or III chemoprevention trials for prostate, breast and colon cancer (Greenwald et al., 2002).

Saco cherry is an old traditional Portuguese variety that has protected geographical indication (PGI) registration according to the EU regulations ("Cova da Beira" cherry). In spite of the powerful antioxidant and antiproliferative effect demonstrated for this variety (Chapter 2, Part II), its acceptance by consumers could be compromised due to the small size and weight of the fruit (Gonçalves et al., 2004; Kappel et al., 1996; Predieri et al., 2004).

In the last years, there has been a growing interest in the recovery of bioactive compounds from natural sources for the development of functional foods. Supercritical fluid extraction (SFE) has been recognized as a promising process for food, pharmaceutical and cosmetic applications as it provides higher selectivity, shorter extraction times and do not use toxic organic solvents (Diaz-Reinoso et al., 2006; Herrero et al., 2006). CO_2 is generally the most desirable solvent for extraction. However, the main drawbacks of CO_2 are its nonpolar and lipophilic nature and its inability to extract compounds of high molecular weight. Thus, the use of suitable cosolvents has been proposed to enhance the solubility of the target compounds and/or to increase the extraction selectivity. Another strategy to overcome this problem is the application of enhanced solvent extraction (ESE). This technique involves the use of CO_2, water and/or organic solvents at elevated temperatures (40-200ºC) and pressures (3.3-20.3 MPa) and has been successfully applied on the extraction of polar solutes (Yuan and Olesik, 1997) and anthocyanins from elderberry pomace (Seabra et al., 2008).

Subcritical fluid extraction, using ethanol as cosolvent, was already used for extraction of phenolic compounds from sour cherry pomace (Adil et al., 2008).

Adil and co-authors used combinations of pressure, temperature, ethanol concentration and extraction time as variables in order to find the optimal conditions for the recovery of total phenolic compounds and antioxidants. SFE has also been shown to be an effective technique for the extraction of perillyl alcohol from natural matrixes, namely orange and citrus peel (Lee et al., 2001; Lee et al., 2000).

Within this context, the aim of this work was to develop a new high-value bioactive product with anticancer properties from *Saco* cherry, in particular from the cherry culls. The methodology employed – multi-step high pressure extraction- comprised a first step with supercritical CO_2 followed by a second ESE step where different mixtures of CO_2 and EtOH were tested. The influence of the polar ESE solvent mixture composition as well as the effect of a supercritical CO_2 treatment were studied concerning extract yield, phenolic content, antioxidant activity and antiproliferative effect in human colon cancer cells. The most promising products were evaluated for their anticancer potential by analyzing the induction of cell cycle arrest. At the end, different processing strategies were adopted to further increase their antiproliferative potential.

2. EXPERIMENTAL PROCEDURE

2.1. MATERIALS

CO_2 industrial grade (99.5%) (Praxair, Madrid, Spain), CO_2 pure grade (99.998%), EtOH 99.5% (Panreac Quimica SA, Barcelona, Spain) and EtOH 96% (AGA, Lisbon, Portugal) were used for extraction experiments. Chemicals and solvents employed for extracts analyses were: *o*-Phosphoric acid (85%, Panreac, Barcelona, Spain), acetonitrile (99.9%, Fisher, UK), ethanol (99.5%, Panreac, Barcelona, Spain), chloroform (99.4%, Merck, Darmstadt, Germany), iodine (99.8%, Sigma-Aldrich, St Quentin Fallavier, France).

Standards used were catechin, epicatechin, chlorogenic acid and perillyl alcohol from Sigma-Aldrich (St. Louis, MO, USA), and quercetin-3-glucoside, rutin, sakuranetin, luteolin, cyanidin-3-glucoside, cyanidin-3-rutinoside all from Extrasynthèse (Genay, France).

Chemicals used for antioxidant activity assays were: 2',2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich (St Quentin Fallavier, France) and disodium fluorescein (FL) (TCI Europe, Antwerp, Belgium). Sodium chloride, potassium chloride and potassium phosphate all from Sigma-Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate from Riedel-de-Haën (Seelze, Germany) were used for phosphate buffer solution preparation (PBS).

All cell culture media and supplements, namely fetal bovine serum (FBS), glutamine and RPMI 1640, were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). Moreover, chemicals used for antiproliferative assays and cell cycle analyses were: dimethyl sulphoxide (DMSO) (99.5%, Panreac, Barcelona, Spain), ribonuclease A (Calbiochem, Darmstadt, Germany), and methylthiazolyldiphenyl - tetrazolium bromide (MTT), Triton X, sodium chloride, propidium iodide and doxorubicin hydrochloride all from Sigma-Aldrich (St Quentin Fallavier, France).

2.2. RAW MATERIAL

Cherries of Saco variety were collected at Cova da Beira, Portugal, between May and June 2008 and stored at -18ºC. Raw material used in all extractions was obtained from all fruit with seeds and stalks. Firstly, raw material was crushed in a knife mill (UFESA, Lisbon, Portugal) followed by dehydratation in a freeze drier (Freeze Dryer Modulyo, Edwards, UK) at -40ºC, in the absence of light. After 72h the raw material was milled in a grinder (Braun, KSM 2, Kronberg, Germany) and stored at -18ºC until the day of the experiments.

2.3. EXTRACTION PROCEDURE

MULTI-STEP HIGH PRESSURE EXTRACTION (MHPE)

These extractions were carried out using the apparatus schematically represented in figure 3.1 (Braga et al., 2008; Seabra et al., 2008).

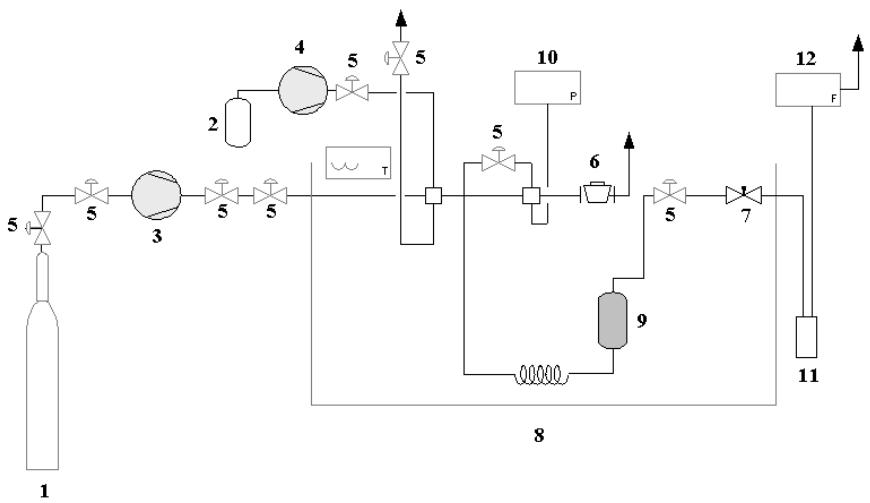

Figure 3.1. Schematic diagram of the employed high pressure extraction apparatus. (Legend: **1-** CO_2 cylinder, **2-** Solvent reservoir, **3-** CO_2 compressor, **4-** High pressure pump, **5-** Valves, **6-** Back- pressure regulator, **7-** Micrometric valve, **8-** Thermostatic water bath, **9-** Extraction cell, **10-** Manometer, **11-** Recovering flask, **12-** Flowmeter)

Liquid CO_2 (99,5%) was delivery to the extraction cell using a high pressure liquid compressor (maximum pressure of 30MPa), and EtOH (99.5%) was delivered by a high pressure liquid pump (L-6200A, Hitachi, Merck Darmstadt, Germany). A stainless steel extraction cell (~20mL) was filled with dried cherry and a filter was placed on both endings of the cell in order to achieve a uniform distribution of the solvent flow and also to prevent line obstructions. Extraction cell was placed into a water bath with temperature controlled by an immersion circulator (± 0.1ºC, DC30, Thermo Haake, Karlsruhe, Germany) and pressure was maintained by a back-pressure regulator (26-1762-24-090, Tescom, Selmsdorf, Germany) and measured by a pressure transducer (C204, Setra, Boxborough, MA, USA). Extracts were recovered in a recovering flask placed in an ice bath and the expanded CO_2 flow was measured by a wet gas meter (DM3C ZE 1411, G.H: Zeal Ltd., London, England). A two-step extraction methodology was employed, comprising: (1) a first CO_2 SFE step in order to remove the low polarity CO_2-soluble compounds (15min static + 60 min dynamic period); (2) a second ESE extraction step, for 90 min, to extract polar compounds and wherein mixtures of CO_2 with EtOH (10-100% v/v) were introduced to the system. The exit tubing line was always cleaned after the second extraction step. Operational conditions of both steps (50ºC and 25 MPa) were selected based on literature information concerning extraction of polyphenols and antioxidants from cherry pomace (Adil et al., 2008) and isolation of perillyl alcohol from natural matrixes (Lee et al., 2001; Lee et al., 2000) using CO_2 or CO_2 and EtOH mixtures. EtOH-containing extracts were concentrated at 40ºC, under vacuum and in absence of light and kept at -18ºC until further analysis. Some assays were performed in triplicate to determine experimental error of the yield values.

SINGLE-STEP HIGH PRESSURE EXTRACTION

One point of experimental design was made without applying a first CO_2 supercritical extraction in order to evaluate the effect of this pre-treatment.

Herein a CO_2:EtOH (80:20 v/v) extraction was employed by 90 plus 60 minutes, using CO_2 99.5% and EtOH 99.5%.

INTEGRATION OF CONVENTIONAL SOLVENT WITH MULTI-STEP HIGH PRESSURE EXTRACTION

Conventional solvent extractions were performed on dried “Saco” cherry prior to multi-step high pressure process.

Solvent extractions were carried out using MetOH, EtOH (96%) and a mixture of EtOH:H_2O (50:50, v/v). Briefly, dried cherries were mixed with solvent (1:20 w/v) and homogenized using a RW20.n agitator (IKA®, WA, USA) at 9000rpm for 60 min. The resulting extract was initially filtered under vacuum through one layer of filter paper (Filter-lab®, Barcelona, Spain) and then concentrated using a rotary evaporator (R-114 Büchi, Postfach, Switzerland) with a water bath (B-480 Büchi, Postfach, Switzerland) at 40ºC in order to remove methanolic or ethanolic fraction. The final concentrates were redissolved in distilled H_2O and liophilized with maltodextrin in a freeze drier (Freeze Dryer Modulyo, Edwards, UK) and kept at -18ºC. All procedures were made in the absence of light and final products contained about 30% (w/w) of maltodextrin. These dried cherry extracts were further submitted to a MHPE as described previously, using CO_2:EtOH (90:10) in the second step.

2.4. CHARACTERIZATION OF EXTRACTS

PERILLYL ALCOHOL DETECTION BY THIN LAYER CHROMATOGRAPHY (TLC)

TLC analysis of cherry extracts was performed using silica gel plates with 254 nm fluorescent indicator (20×20 cm, thickness 0.2 mm) (Macherey-Nagel, Düren, Germany). Chloroform was used as the mobile phase while iodine was applied for detection of perillyl alcohol in cherry extracts. Perillyl alcohol (Sigma-Aldrich, USA) was used as a standard.

IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS BY HPLC

HPLC analyses of cherry extracts were performed with a Surveyor apparatus equipped with a diode array detector (Thermo Finnigan—Surveyor, San Jose, CA, USA) (Bravo et al., 2006). Briefly, separations were performed at 35 ºC with a LiChrospher C18 column (5 µm, 250 mm×4 mm i.d.; Merck AG, Darmstadt, Germany) with a guard cartridge of the same type. A mobile phase constituted by phosphoric acid 0.1% v/v (eluent A) and a mixture of phosphoric acid:acetonitrile:water 5:400:595 v/v/v (eluent B) was used with a discontinuous gradient of 0-20% B (0-15 min), 20% B (15-25 min), 20-70% B (25–70 min), 70% B (70–75 min), 70-100% B (75–85 min) and 100% B (85–90 min), at a flow rate of 0.7 mL/min. Diode array detection was performed between 200 and 800 nm and the data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan—Surveyor, San Jose, CA, USA).

Cherry extracts were diluted in ethanol and microfiltered (0.22µm) before HPLC injection. Identification of compounds was done by comparing retention time, spectra and spiking samples with pure standards. Total chromatographic area at 280nm was used to quantify total phenolic compounds of cherry extracts using a calibration curve of gallic acid. Final results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (dry weight, dw)

IDENTIFICATION AND QUANTIFICATION OF ANTHOCYANINS BY HPLC

HPLC analyses of anthocyanins were carried out according to the procedure described previously (Chapter 2, Part II, section 2.4) and using the same equipment as mentioned for phenolic compounds. The mobile phase used consisted of a gradient mixture of water:formic acid 90:10 v/v (eluent A) and acetonitrile:water:formic acid 40:50:10 v/v/v (eluent B). The following eluents gradient was used: 0- 20% B (0-15min), 20% B (15-25min), 20-70% B (25-70min), 70 %B (70-75 min), 70-100% B (75-85min), 100% B (85–90 min);0-100% A (90-95 min), 100% of eluent A (95-100min). The solvent flow rate was

0.7 mL/min. Acquisition range was set between 190 and 700nm and chromatograms were monitored at 527 nm. Anthocyanin identification in the extracts was done by comparing retention time, spectra and spiking samples with pure standards. Total chromatographic area was used to quantify anthocyanin content of cherry extract using a calibration curve of cyanidin-3-glucoside. Final results were expressed as milligrams of cyanidin-3-glucoside equivalents (C3g) per gram of extract (dw).

IDENTIFICATION OF SAKURANETIN BY LC-DAD-MS/MS

LC-DAD-MS/MS was used in order to identify sakuranetin in cherry extracts. An LC (Alliance, Waters 2695 Separation Module) system with a photodiode array detector (DAD, Waters 2996) set at 360 nm (for monitoring) in tandem with a mass spectrometer (Micromass Quattro Micro API) with a Triple Quadrupole and an ESI source were used.

Chromatographic conditions were as follow: column C18 5 μm 2.1×150 mm (Waters); eluent (A) water-formic acid (99.5:0.5, v/v), (B) acetonitrile. The linear gradient was from 5% (time zero) until 40% of eluent B, in 60 minutes. The flow rate was 0.3 mL/min and the column temperature 35ºC. Samples were kept in the autosampler at 10ºC. Mass range was measured from 100-1000 mau. The spectra were acquired in negative mode; the ESI source conditions were adjusted as follows: capillary 2.50 kV, cone 30.00 V, source temperature 120°C, desolvation temperature 350 °C.

OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

ORAC assay was used to evaluate the antioxidant capacity of the samples towards peroxyl radicals. The assay was carried out following a modified method (Huang et al., 2002) as described previously (Chapter 2, Part II, section 2.5). This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of FL catalyzed by peroxyl radicals generated

from AAPH. All data was expressed as micromoles of trolox equivalents antioxidant capacity per gram of extract (µmol TEAC/g dw).

2.5. CELL- BASED ASSAYS

CELL CULTURE

Human colon cancer cell lines, HT29 and Caco2, were obtained from American Type Culture Collection (ATCC, USA) and Deutsche Sammlung von Microorganismen und Zellkulturen (Barunshweig,Germany), respectively. Both cell lines were grown in RPMI 1640 medium supplemented with 10% of FBS and 2mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC with 5% CO_2 in a humidified atmosphere. All cell culture media and supplements were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK).

CYTOTOXICITY ASSAY

Toxicity assays were performed using completely differentiated Caco2 cells which are a good model of the intestinal barrier. This cell line, originally obtained from a human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005).

Briefly, cells were seeded at a density of 2×10^4 cells/well in 96 well plates and the medium was changed every 48 h. After confluence (about 72 h after seeding), Caco2 cells were incubated with different concentrations of cherry extracts, prepared in ethanol, diluted in culture medium (RPMI medium with 0,5%FBS and 2mM glutamine). Control cells contained the solvent alone. After 24, 48, 72 or 96 hours of incubation, the medium was removed and 100 µL of the colorimetric reagent MTT (0.5mg/mL) was added to each well and

left for 4 h. MTT is reduced to a purple formazan product by mitochondrial reductase enzymes active cell viability in viable cells and therefore the amount of formazan product is proportional to the number of viable cells. Reaction was stopped with DMSO (150µL/well) and formazan was quantified by measurement of the absorbance at 570 nm in a SPECTRAmax^{TM} microplate reader (Molecular Devices Corporation, Sunnyvale, USA).

Results were calculated in terms of percentage of cellular viability relative to control (%). Experiments were performed in triplicate.

ANTIPROLIFERATIVE ASSAY

Cherry extracts were evaluated for their cell-growth inhibiting potential in HT29 cells which are a widely used model for *in vitro* colorectal cancer studies (Ferraz et al., 2005; Gill et al., 2005).

Briefly, cells were cultured in 96-well microplates at a density of 1 x 10^4cells/well. After 24 hours of incubation at 37ºC in 5% CO_2, the medium of each well was replaced by medium containing the cherry extracts prepared in EtOH and diluted in culture medium (RPMI medium with 0.5% FBS and 2mM glutamine). Control cells contained the solvent alone. Cells were allowed to proliferate for 24, 48, 72 and 96 hours and after that cells viability was assayed by MTT assay as reported in cytotoxicity tests. Results were expressed in terms of percentage (%) of cellular viability relative to control (cells without cherry extracts). For some extracts, the amount of sample necessary to decrease 50% of the cellular viability, ED_{50} (effective dose), was also calculated. The experiments were performed in triplicate.

CELL CYCLE ANALYSIS

HT29 cells were seeded in 25 cm^2 culture flasks at a density of 1×10^6 cell/flask. After 24 hours, cells were treated with cherry extracts (0.5 mg/mL) or doxorubicin (125 nM) diluted in culture medium (RPMI medium with 0.5% FBS

and 2mM glutamine) for 4, 24, 48, 72 and 96 hours. Control cells were performed using culture medium, only. At 4, 24, 48, 72 and 96 hours of incubation, cells were trypsinized, washed with cold PBS and centrifuged twice (200g) for 10 minutes. Afterwards, supernatant was discarded and 1 mL of a solution containing propidium iodide (50 mg/L), 1.5% Triton – X (1.5%), ribonuclease A (0.700 U/mL) and NaCl (0.01M) was added to 1×10^6cells. Cell suspensions were incubated for 2 hours at room temperature and then at 4ºC overnight. The samples were sorted by flow cytometer CyFlow Space (Partec, Germany) and cell cycle analysis was done with FlowMax cell cycle platform (Partec, Germany).

3. RESULTS AND DISCUSSION

3.1. CHARACTERIZATION OF EXTRACTS FROM *SACO* CHERRY

Saco cherry culls were dried and further submitted to a high pressure extraction methodology in order to obtain a promising extract with anticancer properties. The methodology employed comprised a first step with supercritical CO_2 and a second ESE step where different mixtures of CO_2 and EtOH (10-100% v/v) were tested (Figure 3.2A). The extracts obtained were identified from A to G according to the extraction process (Table 3.1).

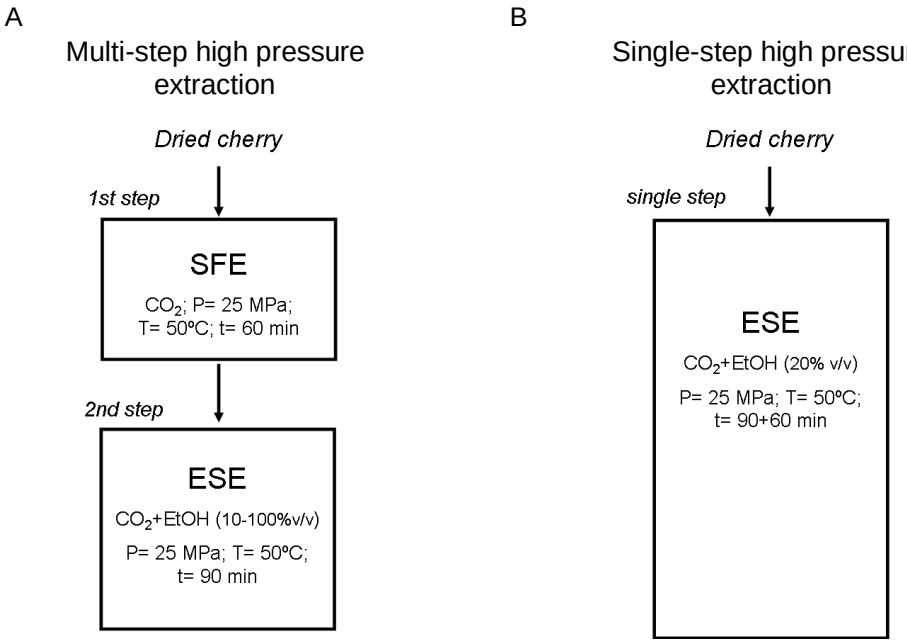

Figure 3.2. Experimental outline used for the multi- (A) and single-step (B) high pressure extractions performed on *Saco* cherry (SFE, supercritical fluid extraction; ESE, enhanced solvent extraction)

All extractions were performed at 50ºC and 25 MPa during 60 plus 90 minutes. The selection of these operating conditions was based on previous studies. According to Lee *et al.* (2001), the extraction conditions that successfully isolate perillyl alcohol from orange and citrus peel were supercritical CO_2 at

50ºC and 20 MPa. Moreover, increasing the operating pressure and adding EtOH as a modifier were reported to enhance the extraction of bioactive compounds from cherry, such as polyphenols (Adil et al., 2008).

Total yields of cherry extracts are presented in table 3.1 and, as can be seen, lower values were achieved in the first step where non polar and lipophilic substances were preferentially extracted. The extract obtained contained lower total polyphenolic content (TPC) and antioxidant activity (AA).

Table 3.1. Multi- and single-step *Saco* cherry extraction at 50±0.1ºC and 25±0.4 MPa: experimental conditions, global yield, composition and antioxidant activity of obtained extracts

Sample	Solvent mixture (CO_2:EtOH)	Yield (%)	POH	TPC	TAC	AA	AA/TPC
Multi-step Extraction							
• ***1st step:*** *Supercritical extraction (extraction time- 60min; solid/solvent ratio- 1: 47 ± 2.4)*							
A	(100:0)	0.53	+	0.55 ± 0.06	nd	44.5 ± 5.47	81
• ***2nd step:*** *Enhanced solvent extraction (extraction time- 90min; solid/solvent ratio- 1: 67 ± 2.6)*							
B	(90:10)	1.53	+	2.50 ± 0.13	nd	181.4 ± 23.7	72
C	(80:20)	3.62	+	1.90 ± 0.10	0.08± 0.01	134.8 ± 10.6	70
D	(60:40)	8.11	○	1.92 ± 0.10	0.33± 0.02	125.7 ± 6.8	65
E	(40:60)	15.15	○	2.46 ± 0.12	0.62± 0.03	120.4 ± 18.3	49
F	(25:75)	20.94	nd	2.30 ± 0.11	0.81± 0.04	121.0 ± 15.4	52
G	(0:100)	28.75	nd	2.87 ± 0.14	0.99± 0.05	169.5 ± 20.3	59
Single-step Extraction *(extraction time- 90(+60 min); solid/solvent ratio- 1:65(44))*							
	(80:20)						
H	90min	3.03	+	2.37± 0.12	nd	189.4± 2.8	80
I	+60 min	1.22	+	1.77± 0.09	0.10± 0.01	166.0± 4.3	94

POH, perillyl alcohol detection by TLC (+, well defined zone detected; ○, less defined zone detected; nd, non-detected); **TPC**, total polyphenolic content determined by HPLC (mg GAE/g dw); **TAC**, total anthocyanin content determined by HPLC (mg C3g/g dw); **AA**, Antioxidant activity (μmol TEAC/g dw)

For the second step, extract yields and the solvent EtOH concentration were directly related (Table 3.1). As expected, EtOH concentration in the solvent mixture affected positively the extraction of polyphenols (Figure 3.3). This fact is related with more hydrogen bonding and dipole-dipole interactions in solvent which increase solubility of phenolic compounds. Additionally, the solubility of polyphenols depends on their polarity (Berna et al., 2001; Chafer et al., 2002; Chafer et al., 2004). In this work, anthocyanins, that are polar and high molecular weight compounds, were only extracted when more than 20% of EtOH was present in the solvent mixture (Figure 3.3). In fact, a red/pink sample colour started to appear on CO_2:EtOH (80:20) extraction onwards confirming the presence of anthocyanins (Figure 3.4). Moreover, the extraction of these compounds was higher when no CO_2 was present in the solvent mixture, which indicates that CO_2 had a negative effect on anthocyanins extraction yield. On contrary to Seabra *et al.* (2008), herein the increase in the solvent density and polarity was more pronounced for anthocyanins extraction than the positive influence of enhanced transport properties of gas expanded liquids including the pH decrease of the solvent mixture.

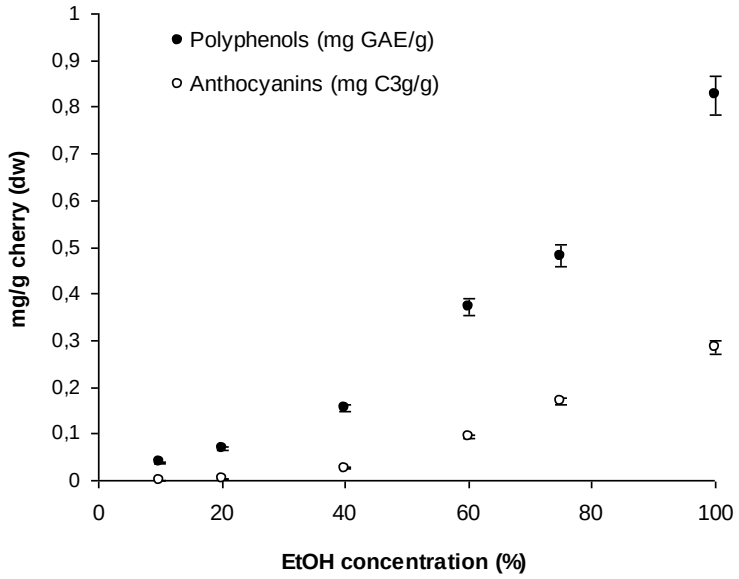

Figure 3.3. Effect of solvent EtOH concentration on polyphenols and anthocyanins extracted from dried *Saco* cherry

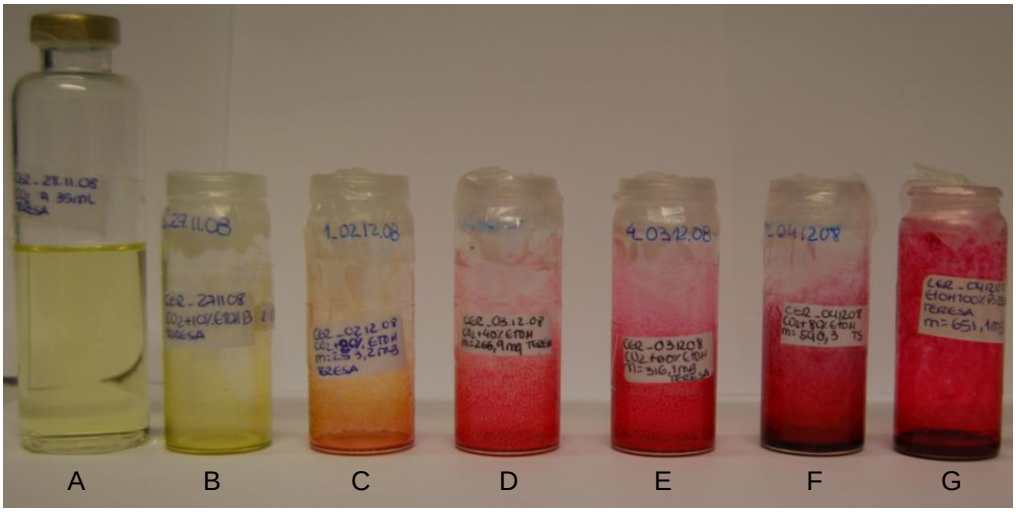

Figure 3.4. Cherry extracts obtained by multi-step high pressure extraction

Among all extracts, G contained the highest phenolic concentration, followed by B. Additionally, for these two extracts, higher antioxidant values were obtained but the latter achieved the best results. In fact, the ratio AA/TPC was higher when lower percentages of EtOH were added to the solvent mixture which indicates that CO_2 enhanced the extraction of highly antioxidant polyphenols. Accordingly, the ratio (AA/TPC) of the extracts obtained with supercritical CO_2 (A) in the first step was also higher (Table 1). For the second step, a higher value was found for extract B decreasing afterwards until extract D. After that, a slightly increase was observed with the increase of EtOH concentration and this is probably due to the presence of higher concentration of anthocyanins in the extracts (Table 3.1), which are powerful antioxidant substances. Figure 3.5 shows the chromatographic profile of all extracts (25 mg/mL) at 280 nm and as it can be seen fewer phenolic compounds were detected in the first fraction of the process. For the second step, some variations in the content were detected between samples. As reported previously, anthocyanins, in particular cyanidin-3-glucoside and cyanidin-3-rutinoside, were only found in extract C onwards being G the product which contained the highest amount of these coloured compounds (Table 3.1).

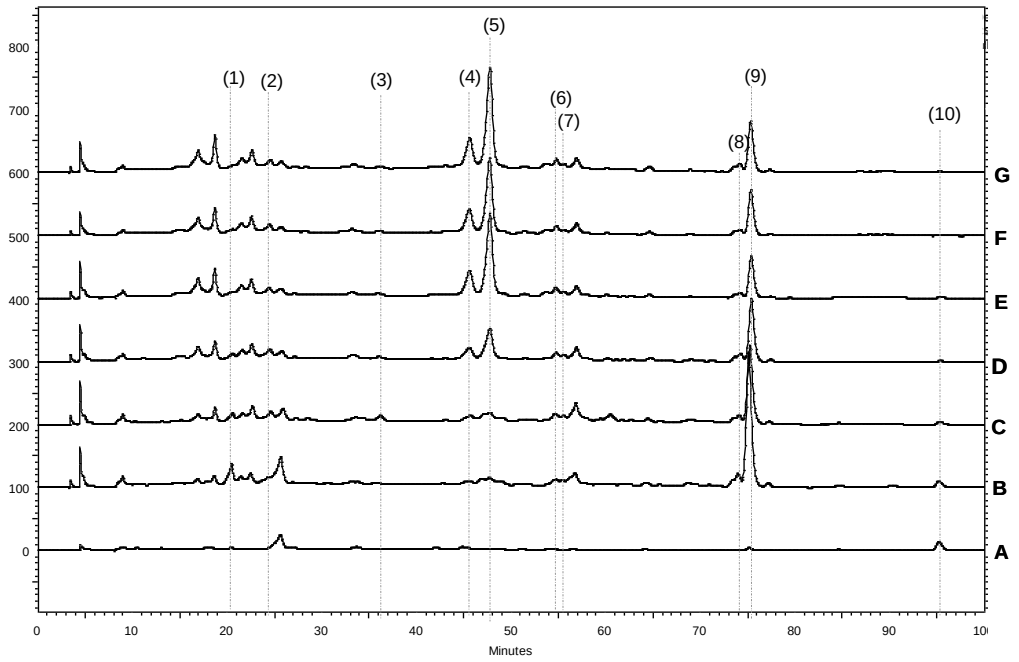

Figure 3.5. Chromatographic profile of cherry extracts (25mg/mL) derived from multi-step high pressure process, using HPLC-DAD at 280nm. (Legend: **1**- neochlorogenic acid; **2**- catechin; **3**- epicatechin; **4**- cyanidin-3-glucoside; **5**- cyanidin-3-rutinoside; **6**- rutin; **7**- quercetin-3-glucoside; **8**- luteolin; **9-** sakuranin; **10-** sakuranetin)

The presence of perillyl alcohol in all cherry extracts was analysed using TLC technique. Among all fractions, only the three extracts obtained with higher percentages of CO_2 (A, B and C) showed a well defined and intense zone at the same retention time as the standard (Table 3.1). As reported by Lee et al. (2000), perillyl alcohol was efficiently extracted from dried Korean orange peel using only CO_2 at supercritical conditions and, when they compared with conventional solvent extraction with methanol, the yield of perillyl alcohol extracted by supercritical technology was about 30 times higher. For F and G no perillyl alcohol was detected whereas for D and E a less defined zone with lower intensity as standard was identified (Table 3.1).

3.2. MULTI- VERSUS SINGLE-STEP HIGH PRESSURE EXTRACTION

In order to study the effect of a supercritical CO_2 pre-treatment, an extraction with CO_2:EtOH (80:20) was directly performed on dried "Saco" cherries during 90 min plus 60min (Figure 3.2B). When considering the total time of extraction (150min), no differences were observed between the global yields achieved in these two types of extractions (Table 3.1). However, the recovery of total phenolic compounds and antioxidant activity from dried cherry was higher in single-step extraction process (Figure 3.6A, B). Herein, an increase of 30% in total polyphenols extracted was obtained in the last hour of the process, indicating that the diffusion period was not reached within 90 min as the raw material still had more soluble phenolic compounds to be extracted (Figure 3.6A). In the first 90 min, the yield of polyphenols extracted (71.8×10^{-3} mg GAE/g cherry dw) was similar with those resulted from the both steps of multi-step extraction ($2.9 + 68.8\times10^{-3}$ mg GAE/g cherry dw).

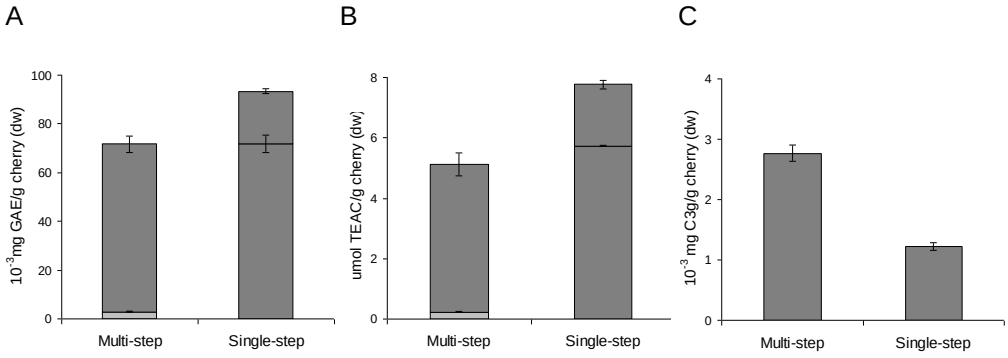

Figure 3.6. Comparison of cherry extracts derived from multi- and single-step high pressure processes: (A) Total polyphenols extracted (B) Antioxidant activity extracted; (C) Total anthocyanins extracted; light grey- CO_2:EtOH (100:0); dark grey- CO_2:EtOH (90:10)

The products derived from the single-step extraction process (H, I) had higher antioxidant activity than those obtained from the multi-step method (A, C), which could be related with their content in polyphenols. As shown in figure

3.7, the formers contained higher concentration of sakuranin (identified by LC-DAD-MS/MS), a glucoside of sakuranetin which is reported to be an effective antioxidant compound (Soares et al., 2005). In contrast to the second part of multi-step extraction, no anthocyanins were extracted within the first 90 min of single-step process (Figure 3.6C). In fact, these polar and high molecular weight polyphenols were only detected in the extract derived from the last process hour (Table 3.1). When comparing both processes, the yield of anthocyanins extracted from cherries was about 2 times higher in the multi-step process (Figure 3.6C). This interesting result could be explained by the fact that, when a pre-treatment with supercritical CO_2 was applied, lipophilic and non polar substances were efficiently removed, making anthocyanins more available for extraction (Yuan and Olesik, 1997).

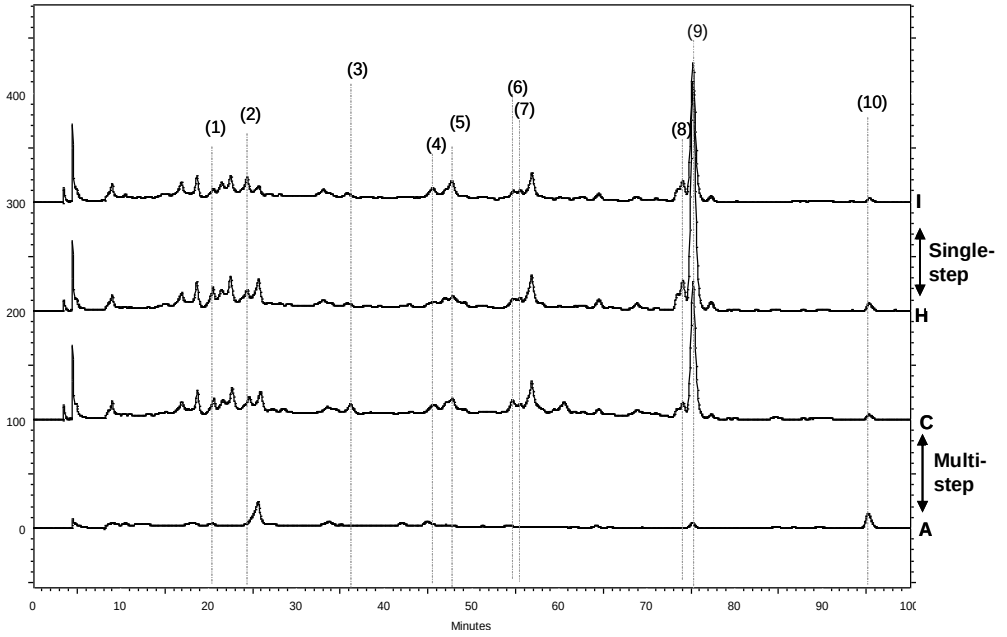

Figure 3.7. Chromatographic profile of cherry extracts (25mg/mL) derived from multi- and single-step high pressure processes, using HPLC-DAD at 280nm. (Legend: **1-** neochlorogenic acid; **2-** catechin; **3-** epicatechin; **4-** cyanidin-3-glucoside; **5-** cyanidin-3-rutinoside; **6-** rutin; **7-** quercetin-3-glucoside; **8-** luteolin; **9-** sakuranin; **10-** sakuranetin)

Extracts derived from the single-step high pressure process were analysed by TLC and perillyl alcohol was detected in both samples (Table 3.1).

3.3. ANTICANCER ACTIVITY OF CHERRY EXTRACTS

In order to examine the antiproliferative effect of extracts, human colon cancer cells (HT29) were subjected to 24, 48, 72 and 96 h of treatment with 0.5 mg/mL of cherry extracts and cell viability was evaluated using MTT assay. It is important to note that this concentration was not cytotoxic in Caco2 cells (data not shown) which are a good model of the human intestinal barrier.

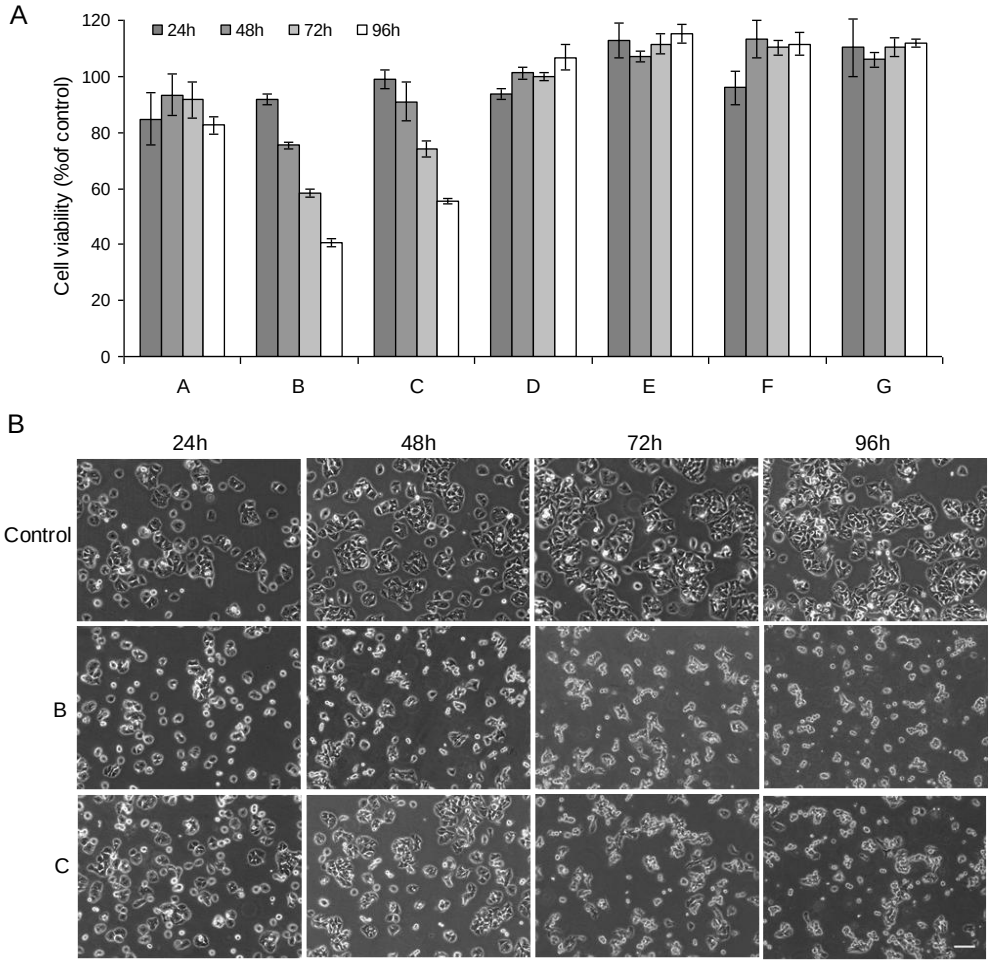

Figure 3.8. Antiproliferative activity of cherry extracts (0.5 mg/mL) in human colon cancer cells (HT29). (A) Cell viability measured by MTT assay; (B) Phase contrast images of cell cultures incubated with extract B and C. Control cultures were performed by incubating cells in standard culture medium with the solvent. Scale bar: 100μm

For antiproliferative assays, only two extracts, namely B and C, inhibited cancer cell growth in a time dependent effect (Figure 3.8). This result could be related to the presence of perillyl alcohol that was already reported to inhibited proliferation of HT29 cells (Crowell et al., 1994). When comparing the extracts derived from multi- and single-step extraction processes, it can be concluded that a pre-treatment with supercritical CO_2 is required to obtain a more concentrated extract with antiproliferative activity (Figure 3.9).

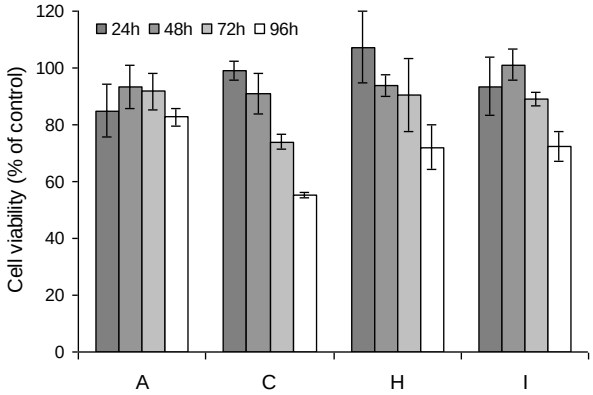

Figure 3.9. Comparison of antiproliferative activity in human colon cancer cells (HT29) of cherry extracts (0.5 mg/mL) obtained from multi- (A,C) and single-step (H,I) high pressure processes.

In order to better understand the antiproliferative effect demonstrated by B and C extracts, flow cytometry was performed to analyse the induction of cell cycle arrest. In this study, HT29 cells were treated with 0.5 mg/mL of cherry extracts and results were compared with doxorubicin, a common drug used in cancer chemotherapy. Results obtained shows that, when cells were treated with both cherry extracts there was an increase of HT29 population in G1 phase (cell growth phase) during time (Figure 3.10A, B). This increase was more pronounced in extract B, accompanied by a decrease in G2/M (cell division phase) population. This effect could be related with the presence of POH, which is reported to induce G1 arrest in a variety of human cell models, including lung, pancreatic and breast cancer cells (Yeruva et al., 2007; Yuri et

al., 2004). On contrary, doxorubicin induced cell cycle arrest in G2/M phase (Figure 3.10C), as mentioned by Chang et al. (2001) using human fibrosarcoma cells. In fact, when comparing cell sizes of all assays, drug treated cells were larger which is characteristic of G2/M phase (Figure 3.10D) (Lloyd et al., 2000). These results are very promising for the use of these cherry extracts as chemotherapeutic agent for colon cancer since it has been demonstrated that potent inhibition of tumour survival is achieved when combining drugs with different cell cycle check points (Chang et al., 2001; Li et al., 1999).

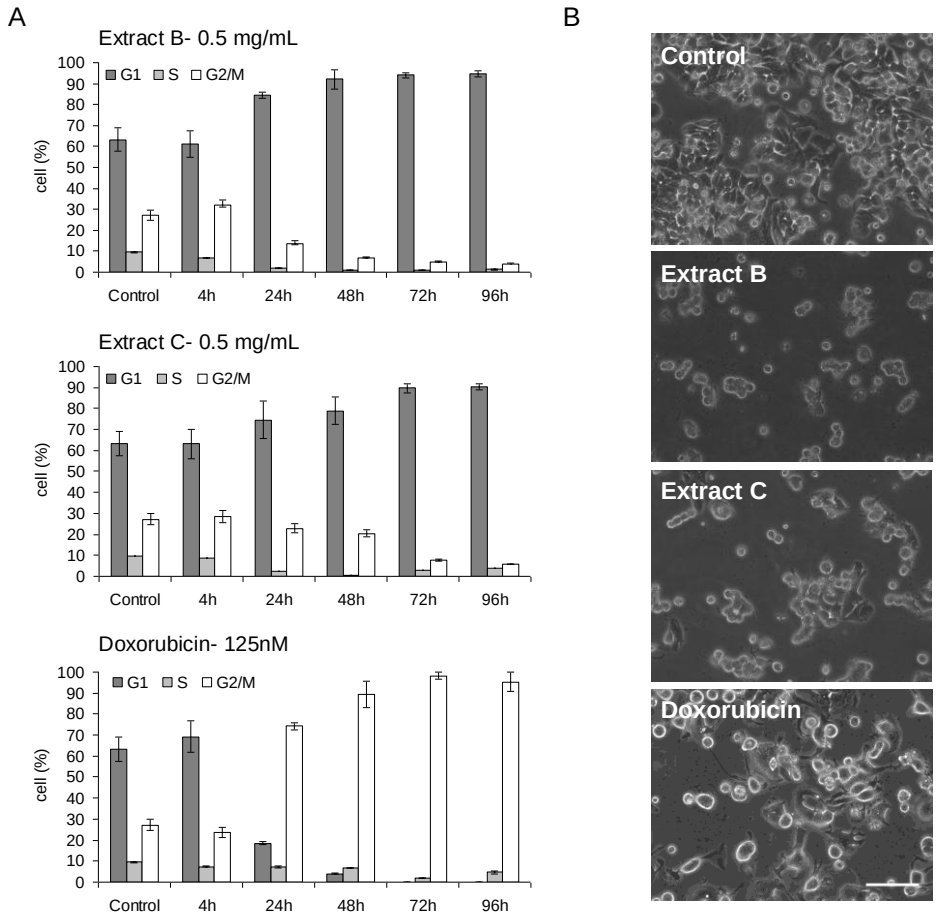

Figure 3.10. Cell cycle analysis by flow cytometry of HT29 cells incubated with cherry extracts (0.5 mg/mL) or doxorubicin (125 nM). Control cultures were performed by incubating cells in standard culture medium with the solvent. (A) Cell cycle distribution; (B) Phase contrast images of cells after 96h of incubation. Scale bar: 100µm

Table 3.2. Effective dose values of cherry extracts on HT29 cells, after 96h of proliferation

Sample	$ED_{50,\ 96h}$ (mg/mL)
Extract B	0.20 ± 0.02
Extract C	0.63 ± 0.03
Saco cherry (dried)	5.4 ± 0.4*

* Chapter 2, Part II (mg/g dw cherry)

Overall, extract B was the most efficient in inhibiting human colon cancer cells growth. The concentration required to reduce 50% of HT29 viability after 96 h of incubation (effective dose value -$ED_{50,96h}$) was found to be lower than in extract C (Table 3.2). When directly compared with fresh Saco cherry (% water content = 82.5% (Gonçalves et al., 2007), extract B is about 150 times more effective. However, it is important to note that the $ED_{50,96h}$ value obtained for doxorubicin was 625 times lower (3.2 ± 0.1 μg/mL).

3.4. PROCESSING STRATEGIES TO OBTAIN EXTRACTS WITH ENHANCED ANTIPROLIFERATIVE ACTIVITY

Aiming to increase the concentration of anticancer compounds in final extracts, two different extraction strategies were adopted in the most promising process- CO_2:EtOH (90:10) extraction after a pre-treatment of raw material with supercritical CO_2 (Figure 3.2A).

ALTERING PURITY OF EXTRACTION SOLVENTS: USING CO_2 (99.998%) AND ETOH (96%)

On a first approach, raw material (dried *Saco* cherry culls) was submitted to a multi-step high pressure extraction using a higher pure CO_2 and less pure EtOH. The resulting extract (designated as J) was analysed for antiproliferative activity in HT29 cells and a higher inhibition was observed

(Figure 3.11). As shown in figure 3.11A, cells incubated with 0.5 mg/mL of this extract, during 96 h, had a more pronounced decrease in cell viability. Moreover, 0.03 mg/mL of extract J induced similar response in cancer cell proliferation as 0.5mg/mL of the extract B (Figure 3.11A). Thus, the use of a better quality of CO_2 improves the functionality of the final product in about 16 times. The extract J also showed a significant antiproliferative effect when the assay was performed for 24 h (Figure 3.11B) which could be related with the higher extraction selectivity of anticancer compounds.

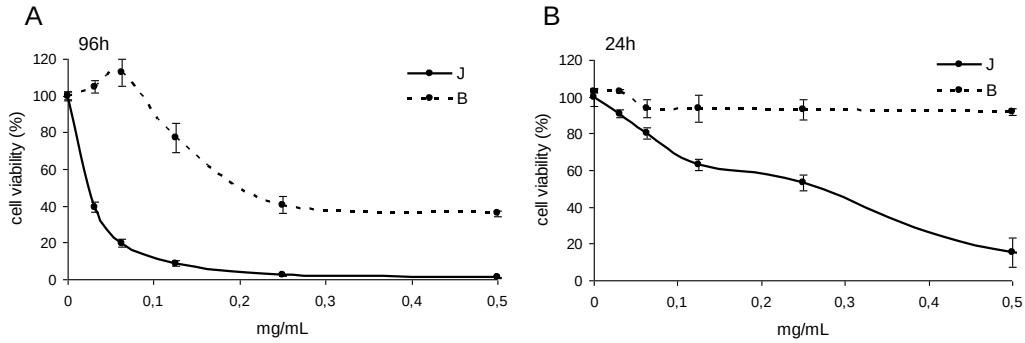

Figure 3.11. Antiproliferative activity of cherry extracts B and J. HT29 colon cancer cells were incubated with cherry extracts and allowed to proliferate for (A) 96 hours and (B) 24 hours

Lee et al. (2000) already reported the use of highly pure CO_2 (99.95%) for the extraction of perillyl alcohol from Korean orange peel. In this work, the presence of perillyl alcohol was also detected in extract J (Table 3.3).

Table 3.3. Characterization of cherry extracts obtained using MHPE or CSE+MHPE: global yields, presence of perillyl alcohol, total phenolic content and antioxidant activity. (Note: MHPEs were performed with CO_2 99.998% and EtOH 96%)

Sample	Extraction Process	**Yield** (%)	POH	TPC	AA
J	MHPE	0.57±0.10	+	1.88± 0.09	50.9±5.5
K	CSE (MetOH) + MHPE	0.10±0.01	++	0.47± 0.02	66.7±13.0
L	CSE (EtOH) + MHPE	0.18±0.02	+	1.07± 0.05	74.4±1.4
M	CSE (EtOH:H_2O (50:50 v/v)) + MHPE	0.13±0.08	++	0.94± 0.04	63.9±24.2

POH, perillyl alcohol detection by TLC (++, well defined zone detected; +, detected); **TPC**, total polyphenolic content determined by HPLC (mg GAE/g); **AA**, antioxidant activity (μmol TEAC/g); **MHPE**, multi-step high pressure extraction; **CSE**, conventional solvent extraction.

In comparison with the results obtained above (section 3.1), the use of CO_2 99.998% and EtOH 96% reduced the global yield of the process (Table 3.3).

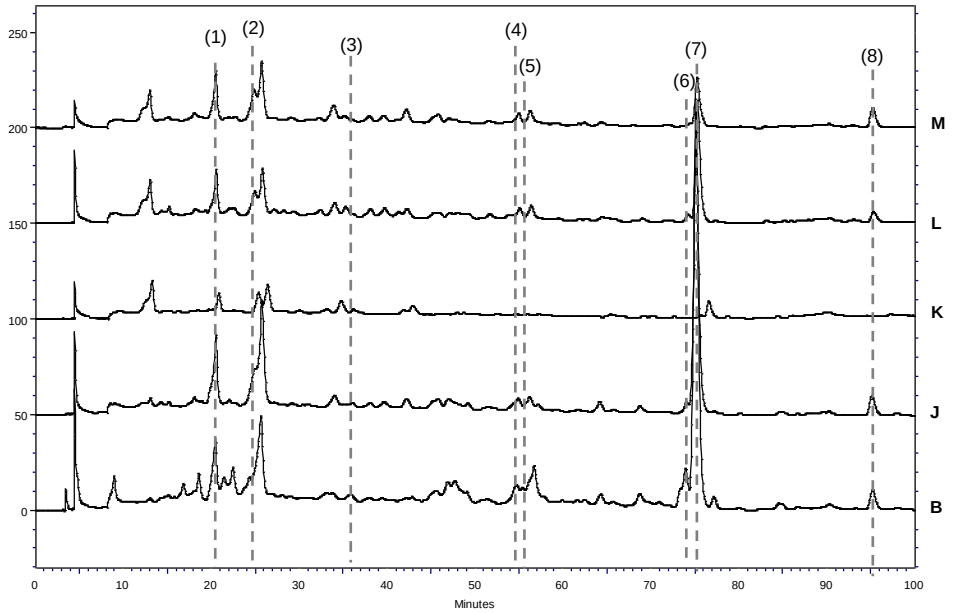

Figure 3.12. HPLC chromatogram at 280 nm of cherry extracts (25 mg/mL). (Legend: **1-** neochlorogenic acid; **2-** catechin; **3-** epicatechin; **4-** rutin; **5-** quercetin-3-glucoside; **6-** luteolin; **7-** sakuranin; **8-** sakuranetin)

The content in polyphenols and antioxidant capacity of the extract J were also lower (Table 3.3 and Figure 3.12) indicating that these compounds were not responsible for the higher antiproliferative activity observed.

INTEGRATING CONVENTIONAL SOLVENT WITH MULTI-STEP HIGH PRESSURE EXTRACTION

Conventional solvent extractions (CSE) were performed in raw material prior to multi-step high pressure treatment (Figure 3.13).

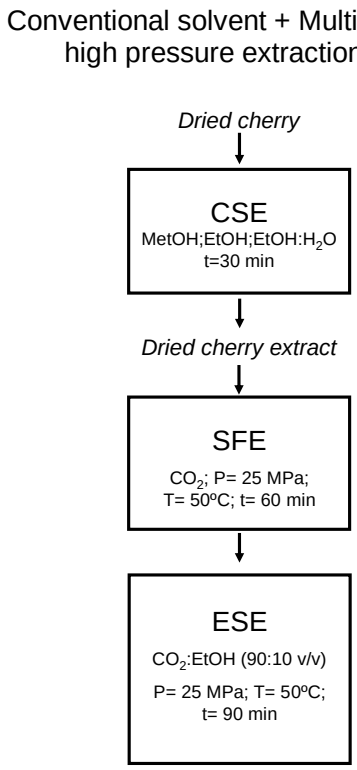

Figure 3.13. Experimental outline used in the integration of conventional solvent and multi-step high pressure extraction of *Saco* cherry (CSE, conventional solvent extraction; SFE, supercritical fluid extraction; ESE, enhanced solvent extraction)

Herein three different solvents were used: MetOH, EtOH and a mixture EtOH:H_2O (50:50 v/v). MetOH was chosen taking into account a previous study related with separation of perillyl alcohol from orange peel (Jung and Row, 1998). In that work, MetOH was pointed as the best solvent for perillyl alcohol extraction due to the higher solubility of this compound in MetOH than in other classes of alcohols. However, the main drawback of MetOH is its toxicity. Therefore, in this work, EtOH was used instead as it is accepted for food and pharmaceutical applications. An EtOH:H_2O (50:50 v/v) extraction was also performed in cherries in order to improve the extraction yields of polyphenols and other antioxidants (Shi et al., 2003).

The products derived from the integrated extraction process were designated as K, L and M according with the solvent used in the conventional extraction – MetOH, EtOH and EtOH:H_2O (50:50 v/v), respectively. Results were compared with extract J.

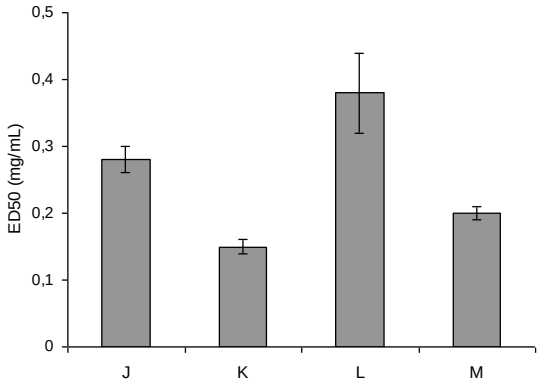

Figure 3.14. Effective dose values (ED_{50}) of J, K, L and M cherry extracts against HT29 proliferation, after 24h of incubation

The best antiproliferative response, in a 24 h assay, was obtained by extract K, followed by extract M (Figure 3.14). These two products were 1.9 and 1.4 times respectively more effective than the extract directly performed from raw material (J) suggesting that a preliminary extraction step with MetOH or EtOH:H_2O (50:50 v/v) is essential to concentrate active compounds. In fact, when analysed for the presence of perillyl alcohol, the extracts K and M showed a well defined zone in the same retention time as the standard (Table 3.3). Moreover, it seems that the powerful antiproliferative effect is not related with the polyphenolic content since these two products contained less phenolic compounds (Table 3.3 and Figure 3.12). On contrary, a pre-treatment with EtOH did not enhance the antiproliferative effect of the final product (extract L) as a higher ED_{50} value was obtainned (Figure 3.14).

It is important to note that the integration of a solvent extraction did not increase the global yield of the process (Table 3.3).

4. CONCLUSIONS

The study reported herein describes the use of a multi-step high pressure process to recover powerful anticancer ingredients from the cull of a traditional Portuguese cherry (*Saco* variety).

By using a supercritical CO_2 extraction in the first step followed by ESE with different mixtures of CO_2 and EtOH (10-100% v/v) it was possible to obtain different extract fractions with different phenolic composition, antioxidant and antiproliferative activities.

The product derived from CO_2:EtOH (90:10 v/v) extraction after a pre-treatment of raw material with supercritical CO_2 during 1 hour, exhibited the highest antiproliferative activity in human HT29 colon cancer cells. In addition, cell cycle arrest was induced in a different check point than doxorubicin, suggesting that it can be used in combination with the drug in cancer chemotherapy.

Processing strategies were also adopted in order to increase the antiproliferative response of this extract. Using CO_2 99,998% and EtOH 96% as extraction solvents in the multi-step high pressure process, the antiproliferative effect was significantly improved by 16 fold. Furthermore, adding a conventional extraction step with MetOH or EtOH:H_2O (50:50 v/v) prior to multi-step high pressure process allowed to obtain cherry extracts with even higher antiproliferative activity.

Perillyl alcohol was pointed to be one of the major responsible compound for anticancer properties of cherry extracts as it was detected in the most promising products, whereas polyphenols, in particular sakuranetin and sakuranin, seemed to be the major contributors of the antioxidant capacity.

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PART II

ISOLATION OF ANTIOXIDANT CONCENTRATES FROM *MALÁPIO FINO* APPLE AND *SACO* CHERRY

ABSTRACT

In the last years, there has been a growing interest in the recovery of antioxidants from natural sources for the development of novel functional ingredients.

In this work, adsorption separation process was applied on *Malápio Fino* apple and *Saco* cherry culls in order to obtain polyphenol rich extracts.

After elution of solvent extracts in a macroporous resin, a fraction much more concentrated (50×) in polyphenols and exhibiting higher antioxidant activity (ORAC and HORAC) was obtained. The main compounds in apple concentrate were catechin, epicatechin, chlorogenic acid, phloridzin and quercetin-3-rhamnoside whereas anthocyanins predominate in the recovered cherry concentrate. The antioxidant activity was also verified at a cellular level using human cell models. Apple and cherry eluted fractions exhibited higher apparent permeability in Caco2 cells (13.9×10^{-6} and 10.8×10^{-6} cm/s) indicating that they are able to cross the intestinal barrier to further deliver their protective effects.

TABLE OF CONTENTS

1. INTRODUCTION

There is increasing evidence that oxidative stress, in particular reactive oxygen species (ROS) and reactive nitrogen species (RNS), are involved in several inflammatory and degenerative diseases. Dietary antioxidants, including polyphenolic compounds, are believed to be effective nutrients in the prevention of these oxidative stress related diseases (Kaur and Kapoor, 2001). Clinical trials and epidemiological studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of inflammation, cardiovascular disease, cancer, and other aging-related disorders (Patil et al., 2009).

Apples (*Malus domestica*) and sweet cherries (*Prunus avium*) are widely consumed and constitute a rich source of antioxidant compounds (Boyer and Liu, 2004; Gonçalves et al., 2004). In Portugal, the annual production of these fruits is about 300,000 and 17,000 ton of apples and cherries, respectively. Between all varieties produced, there are some old traditional fruits only cultivated in specific regions of the country. *Malápio Fino* apple and *Saco* cherry demonstrated to have high phenolic content and antioxidant effect (Serra et al., 2009; Serra et al., 2010). However, the acceptance of these fruits by consumers could be compromised due to the lower sensorial classification of *Malápio Fino* apple (Feliciano et al., 2010) and the small size of the *Saco* cherry (Gonçalves et al., 2004). These fruits could be valorized through their recognition as products with biofunctionality or as rich sources of high added-value ingredients with application in the nutraceutical, cosmeceutical or dietary supplement industries.

Separation by adsorption is one of the most commonly applied processes for the recovery of polyphenols from plant crude extracts and is gaining increasing importance in the food industry (Kammerer, 2007; Kammerer et al., 2006). The application of synthetic resins has several advantages, such as low operation costs, simple handling, and high adsorption capacities for a number of different classes of compound. The use of resins for food

production purposes is regulated by the US Food and Drug Administration and the Council of Europe. The adsorption separation has been already employed for isolation of polyphenols and anthocyanins from apples and tart cherries and results reported show that the extracts obtained have antioxidant and chemotherapeutical activities (Bobe et al., 2006; Schaefer et al., 2006).

Within this context, the aim of this work was to isolate antioxidant-rich products from *Malápio Fino* apple and *Saco* cherry culls (fruit that is not suitable to sell for eating due to small size) using macroporous resins. The resulting products were analysed for their phenolic composition and antioxidant effectiveness using cell-free and cell-based assays and results were compared with previous extracts. Bioavailability assays across human intestinal cell monolayers were also performed in order to access the transport of the antioxidant compounds.

2. EXPERIMENTAL PROCEDURE

2.1. MATERIALS

EtOH 96% (AGA, Lisbon, Portugal), distilled H_2O and food grade macroporous resin Amberlit® XAD-16 were used for extraction experiments.

Chemicals and solvents employed for chemical analyses were: *o*-Phosphoric acid (Panreac, Barcelona, Spain), acetonitrile (Fisher, UK), chloroform (Merck, Darmstadt, Germany), formic acid (Panreac, Barcelona, Spain), methanol (Sigma-Aldrich, St. Quentin Fallavier, France), Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and sodium carbonate (Labscan Dublin, Ireland).

Standards used were: chlorogenic acid from Sigma-Aldrich (St. Louis, MO, USA) and catechin, epicatechin, phloridzin, quercetin-3-rhamnoside, cyanidin-3-glucoside, cyanidin-3-rutinoside all from Extrasynthèse (Genay, France).

Chemicals used for antioxidant activity assays were: 2',2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), caffeic acid, cobalt fluoride tetrahydrate (CoF_2) hydrogen peroxide (H_2O_2), vitamin C and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich (St Quentin Fallavier, France). Disodium fluorescein (FL) was obtained from TCI Europe (Antwerp, Belgium). Phosphate buffer solution (PBS) was prepared in distilled water with sodium chloride, potassium chloride and potassium phosphate (all from Sigma-Aldrich (St Quentin Fallavier, France)) and sodium phosphate dibasic dehydrate from Riedel-de-Haën (Seelze, Germany).

For cellular assays, RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, sodium pyruvate (100x) and MEM non-essential amino acids (MEM NEAA) were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK) whereas MEM medium was obtained from Sigma-Aldrich (St Quentin Fallavier, France). Moreover, chemicals used for cellular antioxidant activity assays were: 2',7'-dichlorofluorescin diacetate (DCFH-DA) and quercetin dehydrated from Sigma-Aldrich (St Quentin Fallavier, France) and dimethyl sulphoxide (DMSO) from Panreac (Barcelona, Spain). Hank's buffered salt

solution (HBSS) was prepared in distilled water with sodium chloride (NaCl), D-(+)-glucose, magnesium chloride (MgCl), potassium chloride (KCl) and sodium bicarbonate ($NaCO_3$) all from Sigma-Aldrich (St Quentin Fallavier, France), and calcium chloride (CaCl, Riedel-de-Haën Seelze, Germany), sodium phosphate di-basic ($Na_2HPO_4{\cdot}2H_2O$, Riedel-de-Haën, Seelze, Germany), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$, Merck, Darmstadt, Germany) and potassium phosphate monobasic (KH_2PO_4, Sigma-Fluka, St Quentin Fallavier, France).

2.2. RAW MATERIAL

Cherries of *Saco* variety were collected at Cova da Beira, Portugal, between May and June 2008 and stored at -18ºC.

Apples of *Malápio Fino* variety were from Mangualde (Portugal) and were harvested in November of 2008.

Raw material used in all extractions was obtained from all fruit with seeds and stalks. Firstly, raw material was crushed in a knife mill (UFESA, Lisbon, Portugal) followed by dehydratation in a freeze drier (Freeze Dryer Modulyo, Edwards, UK) at -40ºC, in the absence of light. After 72h the raw material was milled in a grinder (Braun, KSM 2, Kronberg, Germany) and stored at -18ºC until the day of the experiment.

2.3. PREPARATION OF APPLE AND CHERRY EXTRACTS

SOLVENT EXTRACTS (SE)

Powdered apples and cherries were extracted in the dark with EtOH:H_2O (50:50 v/v) solution (1:20, w/v), for 2 h at room temperature. The extracts were then filtered, centrifuged and the supernatants were concentrated in a rotary evaporator under reduced pressure in a water bath thermostated at 40ºC. Apple and cherry solvent extracts were lyophilized until analysis.

POLYPHENOLS-RICH CONCENTRATES (PRC)

Food grade macroporous resin Amberlite® XAD-16 was used as adsorbent. This resin is allowed to food applications by the U.S. Food and Drug Administration Code of Federal Regulation Title 21 (Scordino et al., 2003).

The necessary preconditioning of the adsorbent was realized by an extensive wash with abundant distilled water to remove salts and impurities, and was then dried at 70ºC for 24 h and then cooled in a desiccator. The dried resin was immersed in ethanol for 12 h. The ethanol was then replaced by distilled water through washing.

The production of apple and cherry polyphenols rich concentrates (PRCs) were performed in batch mode as previously described (Silva et al., 2007). Briefly, aqueous apple and cherry SE were put in contact with resin (80 mg GAE/g resin), inside flaks protected from light. These flaks were submitted to an agitation at 200 rpm during 4 h. After that, supernatant were removed and resins were washed three times with distilled water in order to remove water soluble constituents like sugars, organic acids and minerals. Polyphenols were then eluted with ethanol (96%). The ethanolic fractions were gently concentrated by evaporation, transferred into a water phase and, finally, freeze dried. The resulting PRCs were kept in a cool, dry and dark environment.

2.4. PHENOLIC CHARACTERIZATION

TOTAL PHENOLIC CONTENT

The total concentration of phenolic compounds present in SEs and PRCs was determined according to the modified Folin Ciocalteau colorimetric method (Singleton and Rossi, 1965) as previously described (Chapter 2, Part I, section 2.4). Results were expressed in mg of gallic acid equivalents (GAE) per g of extracts (dry weight- dw).

IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS IN APPLE EXTRACTS BY HPLC

HPLC analyses of apple extracts were performed with a Surveyor apparatus equipped with a diode array detector (Thermo Finnigan—Surveyor, San Jose, CA, USA) (Bravo et al., 2006). Briefly, separations were performed at 35 ºC with a LiChrospher C18 column (5 µm, 250 mm×4 mm i.d.; Merck AG, Darmstadt, Germany) with a guard cartridge of the same type. A mobile phase constituted by phosphoric acid 0.1% v/v (eluent A) and a mixture of phosphoric acid:acetonitrile:water 5:400:595 v/v/v (eluent B) was used with a discontinuous gradient of 0-20% B (0-15 min), 20% B (15-25 min), 20-70% B (25–70 min), 70% B (70–75 min), 70-100% B (75–85 min) and 100% B (85–90 min), at a flow rate of 0.7 mL/min. Diode array detection was performed between 200 and 800 nm and the data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan—Surveyor, San Jose, CA, USA).

Apple extracts were diluted in water and microfiltered (0.45 µm) before HPLC injection. Identification of compounds was done by comparing retention time, spectra and spiking samples with pure standards. Catechin, epicatechin, chlorogenic acid, phloridzin and quercetin-3-rhamnoside were quantified in apple extracts. Coefficients of variation on the HPLC quantifications were <5% and final concentrations were expressed as mg/g dry weight.

IDENTIFICATION AND QUANTIFICATION OF ANTHOCYANINS IN CHERRY EXTRACTS BY HPLC

HPLC analyses of anthocyanins in cherry extracts were carried out according to the procedure described previously (Chapter 2, Part II, section 2.4) and using the same equipment mentioned for phenolic compounds. The mobile phase used consisted of a gradient mixture of water:formic acid 90:10 v/v (eluent A) and acetonitrile:water:formic acid 40:50:10 v/v/v (eluent B). The following eluents gradient was used: 0- 20% B (0-15min), 20% B (15-25min), 20-70% B (25-70min), 70% B (70-75 min), 70-100% B (75-85min), 100% B

(85–90 min); 0-100% A (90-95 min), 100% of eluent A (95-100min). The solvent flow rate was 0.7 mL/min. Acquisition range was set between 190 and 700 nm and chromatograms were monitored at 527 nm. Cyanidin glycosides (cyanidin-3-glucoside and cyanidin-3-rutinoside) were quantified in cherry extracts using standard compounds. Coefficients of variation on the HPLC quantifications were <5% and final concentrations were expressed as mg/g dry weight.

2.5. ANTIOXIDANT ACTIVITY

OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

ORAC assay was used to evaluate the antioxidant capacity of the extracts and vitamin C towards peroxyl radicals. The assay was carried out following the modified method of Huang et al. (2002) using a microplate fluorescent reader (FL800 Bio-Tek Instruments, Winooski, VT, USA) as previously described (Chapter 2, Part I, section 2.5). This assay measures the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH. All data was expressed as micromoles of trolox equivalents antioxidant capacity per gram of extract (µmol TEAC/g dw).

HYDROXYL RADICAL ADVERTING CAPACITY (HORAC)

The HORAC assay was based on a previous method (Ou et al., 2002), modified for the FL800 microplate fluorescence reader as previously described (Chapter 2, Part I, section 2.5). Data were expressed as micromoles of caffeic acid equivalents per gram of dry extract (µmol CAE/g dw).

2.6. CELLULAR ASSAYS

CELL CULTURE

Human colon carcinoma Caco2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and were routinely grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of L-glutamine.

Human hepatocellular carcinoma HepG2 cells were obtained from European Collection of Cell Culture (ECACC, Wiltshire, UK) and were cultured in MEM with 10% FBS, 2mM of L-glutamine, 1% MEM-NEAA and 1% sodium piruvate.

Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

CELLULAR ANTIOXIDANT ACTIVITY

To evaluate the cellular antioxidant capacity of extracts, Caco2 and HepG2 cells were seeded at a density of 2 and 6×10^4 cell/well, respectively, on 96-well plate in 100 μL of growth medium. For Caco2 cells, the medium was changed every 48 hours and the experiments were performed using completely differentiated cells (after reaching confluence- ≈72 hours). For HepG2, cells were incubated for 24 h only. After that, medium was removed and cells were washed twice with HBSS. Triplicate wells were treated for 1 h with 100 μL of extracts plus 25 μM DCFH-DA diluted in HBSS. Then, medium was removed and replaced by HBSS containing 600 μM AAPH or 10 mM H_2O_2. The 96-well microplate was placed into a fluorescence reader (FL800, Bio-Tek Instruments, Winooski, VT, USA) at 37ºC. Emission at 530 ± 25 nm was measured after excitation at 485 ± 20 nm every 5 min for 1 h. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and oxidant (H_2O_2 or AAPH); blank wells contained cells treated with DCFH-DA without oxidant. Quercetin was used as a standard.

Cellular antioxidant activity (CAA) of extracts was quantified according to Wolfe and Liu (2008). After blank and initial fluorescence subtraction, the area under the curve for fluorescence versus time was integrated to calculate the CAA value at each concentration of the fruit as

$$CAA\ unit = 1 - \left(\frac{\int SA}{\int CA} \right)$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve and $\int$CA in the integrated area of the control curve. The median effective concentration (EC_{50}) was determined for the extract from the median effect plot of log(f_a/f_u) vesus log(dose), where f_a is the fraction affected (CAA unit) and f_u is the fraction unaffected (1-CAA unit) by the treatment. The EC_{50} values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. EC_{50} were converted to CAA values, expressed as micromoles of quercetin per g of extract, using the mean EC_{50} value for quercetin from three independent experiments.

BIOAVAILABILITY EXPERIMENTS

For transport studies, Caco2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4 μm pore size, Corning Costar Corp.) in 12-well plates at a density of 1.0×10^5 cell/well. The basolateral (serosal) and apical (mucosal) compartments contained 1.5 and 0.5 mL of culture medium, respectively. Cells were allowed to grow and differentiate to confluent monolayers for 20-24 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2 mM of glutamine and 1% of streptomycin+penycilin) three times a week for 24-28 days. Transepithelial electrical resistance (TEER) of cells grown in Transwell was measured using EVOM™ voltmeter (WPI, Berlin, Germany). Only monolayers with a TEER value higher than 500 Ωcm^2 were used for experiments.

Medium was removed and, after washed with HBSS (pH= 7.4; T= 37ºC), PRC extracts diluted in HBSS (1 g/mL) were added to the apical side of the cells. Transepithelial transport was followed as a function of time. At 15, 30, 60, 120 and 180 min of incubation, samples were collected from the basolateral compartment and immediately frozen until analysis of total polyphenols.

Apparent permeability coefficients (P_{app}) of PREs were calculated for 1 h assay, using the following equation:

$$P_{app} = \frac{V}{AC_0}\frac{dC}{dt}$$

where V is the volume of the solution in the receiving compartment, A is the membrane surface area, C_0 is the initial polyphenols concentration in extract presented in the donor compartment and dC/dt is the change in concentration in the receiver solution over time (Walgren et al., 1998).

3. RESULTS AND DISCUSSION

3.1. YIELD, TOTAL POLYPHENOLIC CONTENT AND COMPOSITION OF PRCS

In this study, macroporous adsorption resin Amberlite XAD16 was used to recover polyphenols from *Malápio Fino* apple and *Saco* cherry crude extracts as described above (Section 2.3 of this Part). The products obtained (Figure 3.15) were analysed for their phenolic content and results are shown in figure 3.16 and summarized in tables 3.4 and 3.5.

Figure 3.15. Picture of polyphenols-rich concentrates. A- Apple PRC, B- Cherry PRC

Polyphenol-rich concentrates (PRCs) presented higher phenolic content than solvent extracts (SEs), and this improvement was approximately 55 and 42 times for apple and cherry, respectively.

Despite the similar total phenolic content found for both PRCs, their composition diverged greatly as they were derived from different natural sources. The main compounds presented in apple PRC were catechin, epicatechin, chlorogenic acid, phloridzin and quercetin-3-rhamnoside whereas antocyanins were the major compounds identified in cherry PRC (Tables 3.4,

3.5). All these compounds are reported to be strong antioxidants (Boyer and Liu, 2004; Lila, 2004) and enriched in PRC extracts.

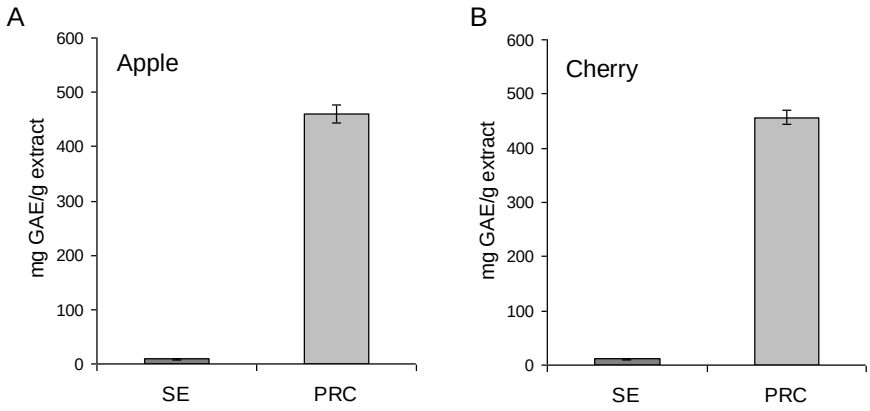

Figure 3.16. Total phenolic content of polyphenols-rich concentrates (PRCs) and solvent extracts (SE). (A) Apple; (B) Cherry.

Table 3.4. Yield and phenolic content of apple extracts (**SE**, solvent extract; **PRC**, Polyphenol Rich Concentrate)

	SE	PRC
Yield (%)	39	0.3
Polyphenols (mg/g)		
Total	8.4	461.2
Catechin	0.06	16.16
Chlorogenic acid	1.37	89.89
Epicatechin	1.48	102.53
Phlorizin	0.13	13.57
Quercetin-3-rham	0.04	5.19

Table 3.5. Yield and phenolic content of cherry extracts (**SE**, solvent extract; **PRC**, Polyphenol Rich Concentrate)

	SE	PRC
Yield (%)	69.5	0.6
Polyphenols (mg/g)		
Total	11.0	456.9
Cyanidin-3-glucoside	0.57	26.82
Cyanidin-3-rutinoside	2.44	114.80
Peonidin-3-glucoside	0.09	4.83

The yield of the process, calculated as grams of products per 100g of raw material (dried apple or dried cherry) was 100× lower in PRCs (Tables 3.4, 3.5).

3.2. ANTIOXIDANT ACTIVITY

In order to access the antioxidant effectiveness of apple and cherry PRCs, two chemical assays were performed: ORAC and HORAC. As already mentioned, these assays measure two different but equally important aspects of antioxidant properties- radical chain breaking and radical prevention. The HORAC primarily reflects metal chelating radical prevention ability, and the ORAC reflects peroxyl radical absorption capacity (Ou et al., 2002).

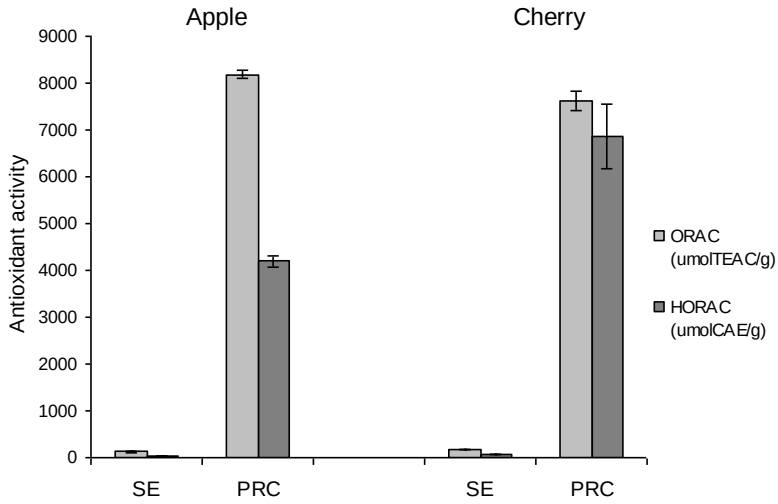

Figure 3.17. Antioxidant activity of apple and cherry extracts (ORAC and HORAC values)

Figure 3.17 shows that PRCs have higher values of ORAC and HORAC than conventional extracts. Cherry PRC exhibited the highest HORAC effect which could be related with the higher content in anthocyanins. In fact, anthocyanin-rich fruits have been reported to have higher HORAC values than apples (Ou et al., 2002). Table 3.6 shows the ORAC values obtained for apple and cherry PRCs, synthetic vitamin C and commercial extracts.

Table 3.6. ORAC values of PRCs, vitamin C and commercial extracts

Product	**ORAC value** (μmol TEAC/g)
Apple PRC	8187 ± 74
Cherry PRC	7611 ± 213
Vitamin C	3220 ± 312
Phytonutriance® apple extract (AF POMM 9080) (80%polyphenols)	8250*
Phytonutriance® bilberry extract (AF MYRT 9025) (25%anthocyanins)	5130*

* http://www.diana-naturals.com/

The results demonstrated that both apple and cherry PRCs are more effective than vitamin C. When compared with commercial extracts, PRCs can be pointed as promising antioxidant products. Cherry PRC has lower content in anthocyanins than Phytonutriance® bilberry extract but exhibited higher antioxidant effect (Tables 3.5, 3.6). Apple PRC has the same ORAC value as Phytonutriance® apple extract, although the phenolic content was 2 times lower (Tables 3.4, 3.6). Overall, these results suggest that polyphenols of PRC are more powerful antioxidants than those present in commercial extracts. This could be related with the raw material used for PRC preparations as the fruit varieties selected for this study demonstrated to have higher antioxidant potential than other apples or cherry cultivars (Serra et al., 2009; Serra et al., 2010).

The antioxidant capacity of apple and cherry PRCs was further characterized using cell-based assays. The human Caco2 and HepG2 cell lines were selected as they are well recognized cell models for antioxidant research (Baker and Baker, 1992; Lima et al., 2006; Liu and Finley, 2005).

Figure 3.18 demonstrates the protective effect of apple and cherry PRCs against H_2O_2 and AAPH induced oxidative stress in Caco2 and HepG2 cells. For both cases, the highest values were achieved for HepG2 incubated with H_2O_2.

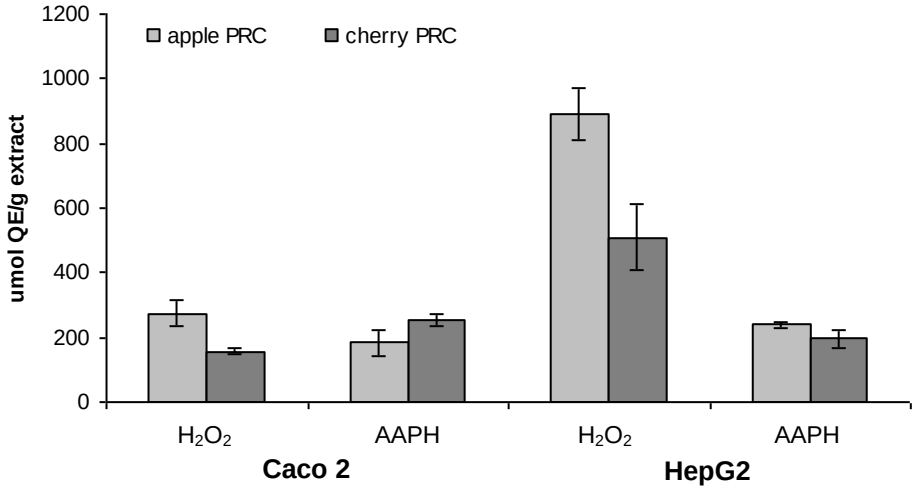

Figure 3.18. Cellular antioxidant activity of apple and cherry PRCs

In the case of H_2O_2 induced oxidative stress, apple PRC exhibited the highest cellular antioxidant activity (CAA) value which could be related with their phenolic composition. In fact, when compared with other polyphenols, anthocyanins demonstrated to have lower cellular uptake due to their poor lipophilic properties (Yi et al., 2006).

Table 3.7. Cellular antioxidant quality of PRCs and fruits in the cellular antioxidant assay performed in HepG2 cells with AAPH

Product	**Cellular Antioxidant Quality** (μmol QE/ 100μmol of phenolics)
Apple PRC	8.8 ± 0.4
Cherry PRC	7.3 ± 1.0
apple	2.4 ± 0.2*
cherry	3.1 ± 0.5*

* Wolfe et al. (2008)

Additionally, PRC extracts showed to be 1000 times more effective than fresh fruits. Wolfe et al. (2008) evaluated CAA of common fruits using HepG2 cells and AAPH as stressor, and reported values of 27.4 ± 4.1 and 21.9 ± 4.0

μmolQE/100g of fresh cherry and apple, respectively. Moreover, PRCs demonstrated higher cellular antioxidant quality (measure of the cellular antioxidant activity provided by 100 μmol of phenolics) (Table 3.7).

3.3. BIOAVAILABILITY STUDIES

To evaluate if PRCs can cross the intestinal barrier, bioavailability assays were performed using a Caco2 cell model (Figure 3.19). It has been reported that these cells can undergo spontaneous differentiation in culture conditions and exhibit the characteristics of mature enterocytes (Hidalgo et al., 1989).

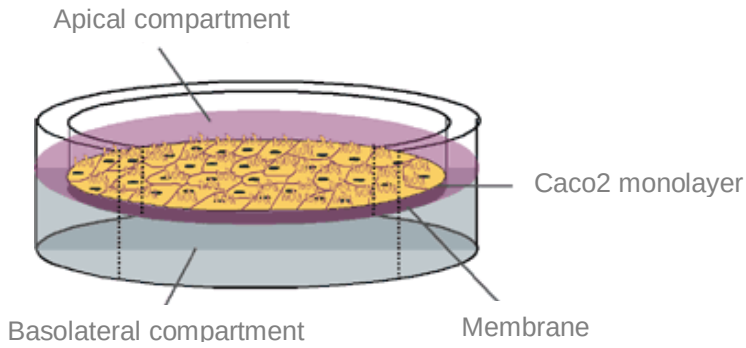

Figure 3.19. Bioavailability in vitro test: Caco2 cell model. The cell surface facing the top medium develops a brush border that resembles the luminal membrane of the intestinal epithelium. The cell surface attaching to the permeable membrane and facing the bottom medium develops into the basolateral membrane (www.cacoready.com/pro_0102.html)

Results obtained demonstrated that popyphenols of PRCs were transported through the Caco 2 cell monolayers (Figure 3.20). In the first 15 min of incubation, 10.5% and 6.6% of polyphenols of apple PRC and cherry PRC, respectively, reached the basolateral side. It should be noted that, in all assays, the integrity of cell monolayer was confirmed by transepithelial electrical resistence (TEER) measurements (data not shown).

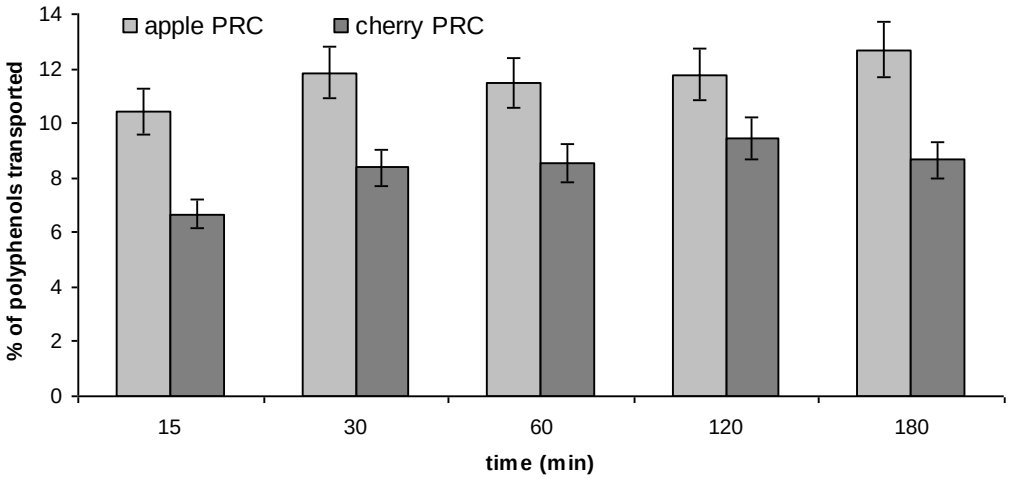

Figure 3.20. Transport of polyphenols from apple and cherry PRCs through the Caco2 cell monolayers

For all time points the fraction of transported polyphenols was higher in apple PRC indicating that polyphenols from apple are more bioavailable than those from cherry. As reported previously, the poor lipophilic properties of anthocyanins compromised their transport trough Caco2 cell monolayers (Yi et al., 2006).

Table 3.8. Apparent permeability values in Caco2 cell monolayers

Product	**Papp** (10^{-6} cm/s)
Apple PRC	13.9
Cherry PRC	10.8
Quercetin	0.5[a]
Phytonutriance® apple extract (AF POMM 9080) (80%polyphenols)	4.18[b]
Phytonutriance® bilberry extract (AF MYRT 9025) (25%anthocyanins)	6.57[b]

[a] Walgren et al., 1998; [b] http://www.diana-naturals.com/

When compared with commercial extracts and quercetin, PRCs exhibited higher apparent permeability (P_{app}) (Table 3.8). Moreover these values were clearly above 2.0×10^{-6}cm/s, which is a threshold value for a bioavailable product (Walgren et al., 1998). Overall, the results showed that apple and cherry PRC are bioavailable in Caco2 model, indicating that they can cross the intestinal barrier and consequently enter the blood stream and reach tissue and organs.

4. CONCLUSIONS

This study describes the use of adsorption process to recover powerful antioxidant ingredients from *Malápio Fino* apple and *Saco* cherry culls.

Using a macroporous resin it was possible to develop new apple- and cherry-based products enriched in polyphenols (40-50% w/w) with powerful antioxidant activity. Moreover, these extracts showed to be bioavailable in a Caco2 cell model assay, indicating that they can cross the intestinal barrier and further deliver their protective effects.

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6. ACKNOWLEDGEMENTS

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CHAPTER 4

GENERAL DISCUSSION AND FINAL CONCLUSIONS

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1. GENERAL DISCUSSION AND FINAL CONCLUSIONS

The advances in understanding the relationship between nutrition and health resulted in the development of the "functional foods concept", a novel approach aiming at obtaining "optimal health *status"* by promoting the well-being and, possibly, reducing the risk of disease. Functional foods are basically natural foods or enriched- foods containing bioactive components that, when consumed regularly exert a specific health-beneficial effect beyond their nutritional properties.

This thesis presents new insights of the health promoting effects of traditional Portuguese apples and cherries that are only cultivated in specific regions of the country. *Saco* cherry and *Bravo de Esmolfe* and *Malápio Fino* apples were identified as powerful antioxidant fruits showing high antiproliferative activity in human colon and gastric cancer cell models. This activity is mainly related with the presence of bioactive compounds, namely polyphenols. *Bravo de Esmolfe* and *Malápio Fino* apples are rich sources of catechin, a powerful antioxidant compound that is recognized to be the major responsible of the health benefits associated to green tea (Frei and Higdon, 2003) and *Saco* cherry presents high amounts of anthocyanins, which are the compounds associated with the red colour of fruits and, more importantly, the main contributors of the biological activities evaluated. Therefore, these fruits can be recognized as products with biofunctionality (natural functional products) or as rich sources of high added-value ingredients with promising application in food, pharmaceutical or cosmetic industry.

The market for bioactive natural ingredients is currently one of the most attractive markets; this is essentially due to the consumer's increasing demand for health-promoting products. In the past few years, many food bioactive constituents have been commercialized in the form of pharmaceutical products (nutraceuticals), cosmetics (cosmeceuticals or

nutricosmetics) or as food additives such as powders and concentrates for the enrichment of products (functional enriched-foods). The development and marketing of these products is rather complex, expensive and risky, as special requirements should be answered. In particular, consumer acceptance has been recognized as a key factor to make a product successful in the market. Not only the product should be in an adequate form so that the consumers could easily accept but also it is necessary to explore which diseases consumers are concerned about. According to survey, primary health concerns among consumers are cardiovascular diseases, stress, high blood pressure, malignant tumour diseases of the digestive system, arthritis and obesity (Hilliam, 1998; Van Kleef et al, 2002; Van Kleef et al., 2005).

In this thesis efforts were directed to develop a promising anticancer agent that can be used as nutraceutical. This product was obtained from *Saco* cherry culls (fruit that is not suitable to sell for eating due to appearance defects, including small size) using a safer and environmental friendly technology. The methodology applied consists in pre-treatment of raw material with supercritical CO_2 followed by a high pressure extraction with CO_2 and ethanol (90:10 v/v), in order to obtain a final product containing perillyl alcohol, a well recognized natural anticancer compound. Additionally, to support health-enhancing claims, *in vitro* studies that investigate the anticancer activities were performed. The product obtained presents higher antiproliferative effect in human colon cancer cells, being 150 times more effective than fresh fruit. Moreover, it induces cell cycle arrest in a different checkpoint than doxorubicin (a well-known anticancer active principle) suggesting that cherry extracts can be used in combination with this drug in cancer chemotherapy.

Different strategies could be also adapted to further increase the antiproliferative activity of the final product. By combining different solvents purity grade and integrating a conventional solvent extraction step (with methanol or ethanol:water) prior to supercritical CO_2 treatment, the

antiproliferative effect of cherry extract can be significantly improved up to 32 fold.

Processing material with high pressure solvents is a proven and industrially applied technology (e.g. decaffeination of coffee beans, de-alcoholization of beverages, removal of pesticides and essential oil extraction). The use of high pressure solvents for the extraction of bioactive compounds from plant sources offers several advantages over conventional techniques, including higher extraction selectivity and lower extraction time. Moreover it is an environmentally friendly process (clean technology) as it uses minimal quantities of organic solvents. All the solvents employed in this work are non toxic and thus the final product is allowed for food and pharmaceutical applications. However, the investment and processing costs associated to this technology are relatively high; reported costs for production rates around 10,000 ton/year of solid feed are in the range of 0.7 €/kg of feed (Brunner, 2005). To overcome this drawback and make costs of high pressure extraction process competitive, it is essential that the product to be extracted has a recognized high added value. In this work, a potential natural chemotherapeutic agent was developed. Future studies using animal models should be performed to validate the anticancer effect of the extract.

Polyphenols-rich concentrates were obtained also from *Saco* cherry culls and *Malápio Fino* apple in order to develop powerful antioxidant powders for future and innovative applications in nutraceuticals formulations, enriched functional foods and drinks, or even in anti-aging cosmetic products.

Apple and cherry powders are 100% natural and present about 50% (w/w) of polyphenols. The main compounds identified in apple powder are catechin, epicatechin, chlorogenic acid, phloridzin and quercetin glycosides which are well known for their antioxidant activity and other interesting health properties likewise anti-aging, inhibition of sugar absorption, draining virtues, anti-inflammatory and suncare. In cherry powder anthocyanins are predominate (≈

15% w/w) and thus this product can be used as more than a natural colouring agent due to the array of health promoting effects associated to these polyphenols (antioxidant, prevention of LDL oxidation, anti-inflammatory and anticancer properties). Both products exhibit higher antioxidant activity than other reference compounds such as vitamin C. Moreover, they are bioavailable which means that, when taken orally, the main bioactive compounds of powders are able to cross the intestinal barrier to further deliver their protective effects in organs and tissues.

There are similar natural antioxidant ingredients derived from apple and bilberry (Phytonutriance®) already available in the market. When compared to these products, the active ingredients present in *Malápio Fino* apple and *Saco* cherry powders are more powerful antioxidant substances and thus can be considering as promising active ingredients to be commercialized. In fact, the raw materials used in these products preparation are traditional Portuguese fruits particularly rich in antioxidant substances. Moreover, the applied process, namely adsorption technology, is gaining increasing importance in the food industry (Kammerer and Carle, 2007) due to the several advantages associated with the application of synthetic resins (low operation costs, easily scale-up, simple handling and high adsorption capacities for a large number of compounds).

Overall, this thesis provides new insights for the health promoting benefits of traditional Portuguese fruits. The results obtained are very encouraging, demonstrating that *Saco* cherry and *Bravo de Esmolfe* and *Malápio Fino* apples present higher bioactivity than other commonly consumed exotic cultivars. It is expected that these fruits would be valorized through their recognition as products with biofunctionality thus positively impacting on their productivity and the local economy. *Bravo de Esmolfe* is a sweet apple and very appreciated by consumers, being easily included in a healthy and "functional" diet. However, due to the lower sensorial classification of *Malápio*

Fino (Feliciano et al., 2010) and the small size of *Saco* cherry (Gonçalves et al., 2004), their acceptance by consumers could be compromised. Alternatively, these fruits can be used as sources of high added-value ingredients with application in the nutraceutical, cosmeceutical or dietary supplement industries.

Hopefully, in the near future, studies in humans will be performed since preliminary animals' experiments shown that *Bravo de Esmolfe* apple reduced rat serum levels of triglycerides and total cholesterol, suggesting that this variety may have a protective effect against cardiovascular diseases (Rocha et al., 2010; Serra et al., 2009).

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APPENDIX

APPENDIX A- EPR spectra of traditional and exotic Portuguese fruits

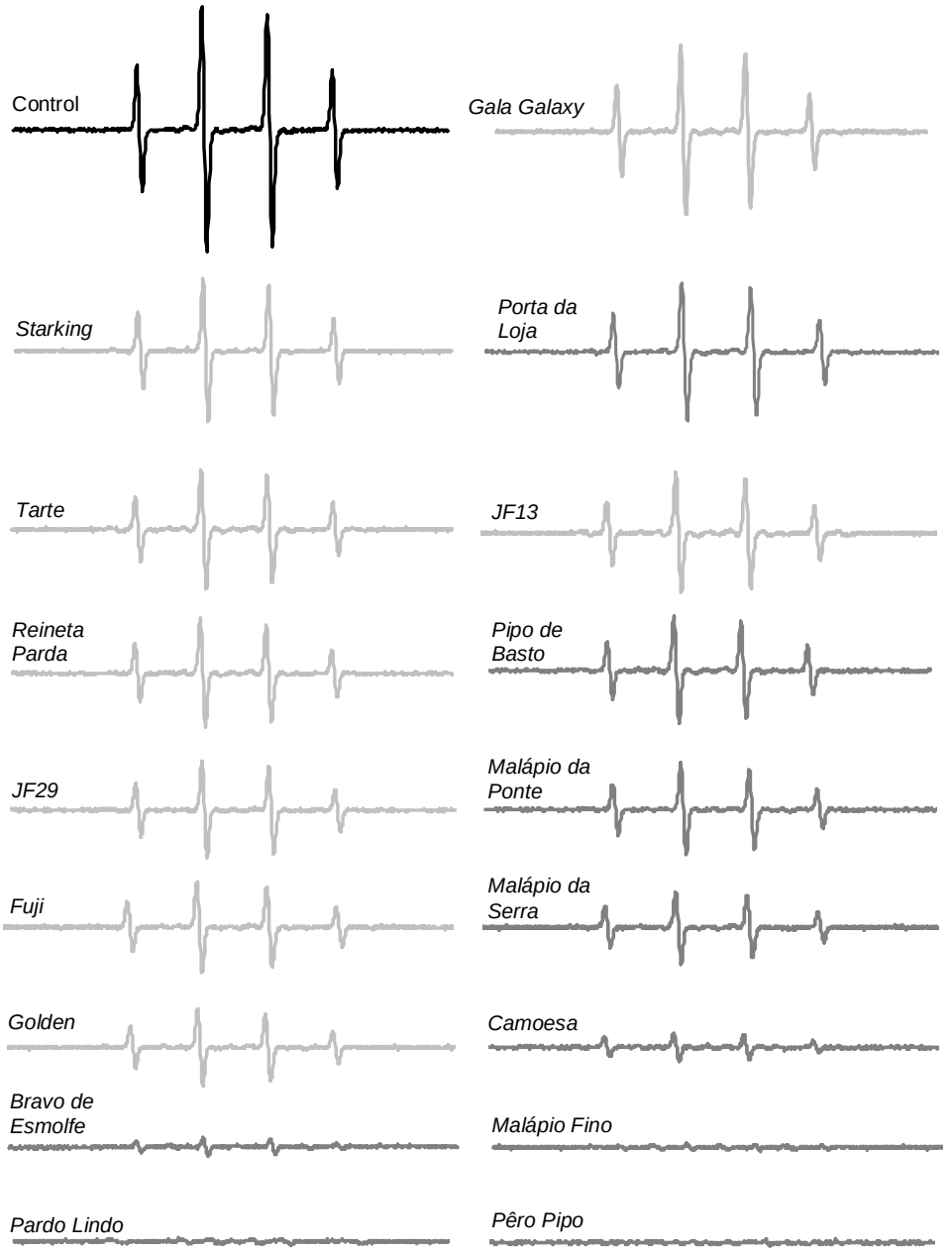

Figure A.1. EPR spectra of 17 apple varieties (— traditional apples; — exotic apples) with a concentration of 0.5g/mL

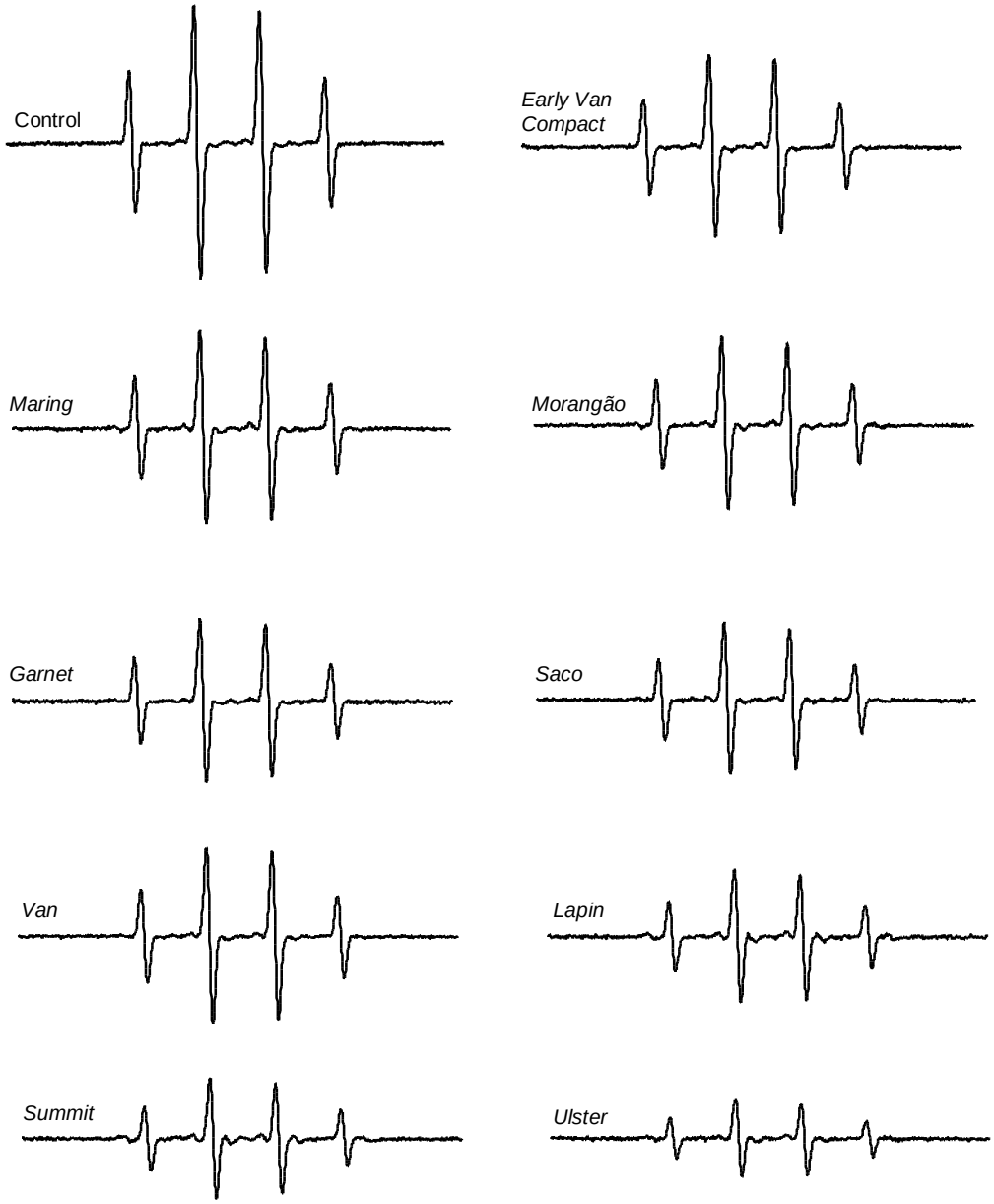

Figure A.2. EPR spectra of nine cherry extracts with a concentration of 125 mg dw/mL

APPENDIX B- HPLC chromatograms of traditional and exotic Portuguese fruits

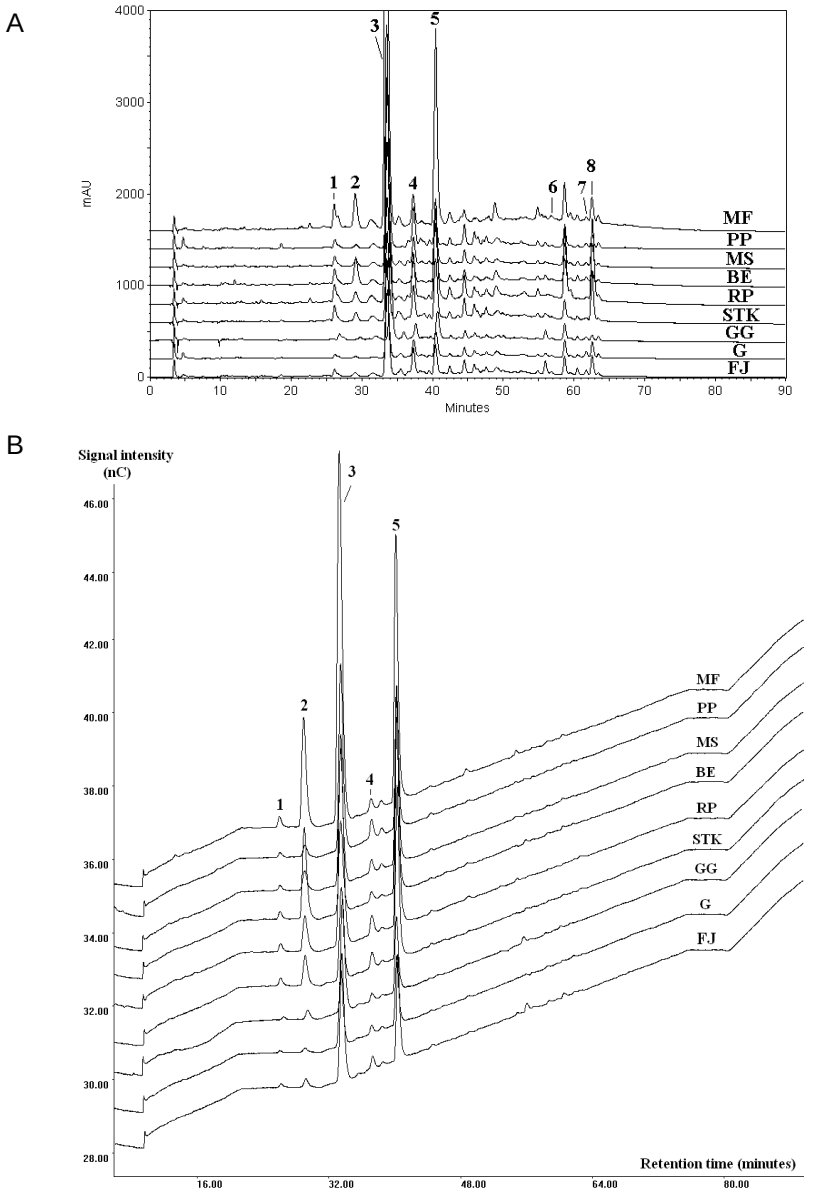

Figure B.1. Chromatographic profiles of apple extracts (2 g/mL) from traditional and exotic apple varieties. A- HPLC-DAD at 280 nm; B- HPLC-ED. Legend: **1**-procyanidin B1; **2**-catechin;**3**- chlorogenic acid; **4**- procyanidin B2; **5**- epicatechin; **6**-quercetin-3-glucoside; **7**-kaemferol-3-glucoside + quercetin-3-rhamnoside; **8**- phloridizin + quercetin-4-glucoside (**MF**, *Malápio Fino*; **PP**, *Pêro Pipo*; **MS**, *Malápio da Serra*; **BE**, *Bravo de Esmolfe*; **RP**, *Reineta Parda*; **STK**, *Starking*; **GG**, *Gala Galaxy*; **G**, *Golden*; **FJ**, *Fuji*)

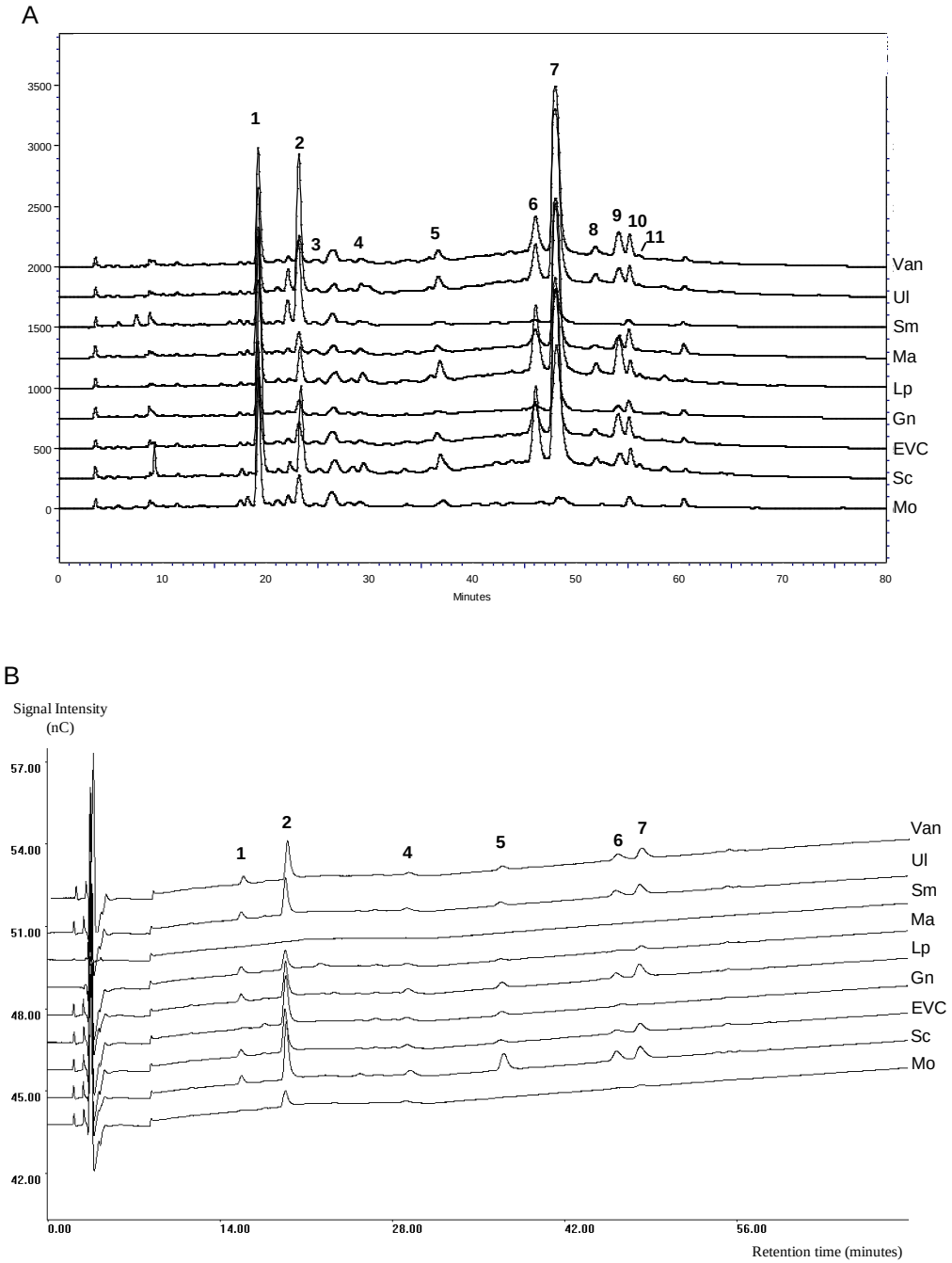

Figure B.2. Chromatographic profiles of cherry extracts extracts (0.2 g/mL) from traditional and exotic cherry varieties. A- HPLC-DAD at 280 nm; B- HPLC-ED. Legend: **1**-p-coumaroylquinic acid; **2**- neochlorogenic acid; **3-** catechin; **4**- chlorogenic acid; **5**- epicatechin; **6**- cyanidin-3-glucoside; **7-** cyanidin-3-rutinoside; **8**- pelargonidin-3-rutinoside + peonidin-3-glucoside; **9**- peonidin-3-rutinoside; **10**- rutin; **11-** quercetin-3-glucoside(**Mo**, *Morangão*; **Sc**, *Saco*; **EVC**, *Early Van Compact*; **Gn**, *Garnet*; **Lp**, *Lapin*; **Ma**, *Maring*; **Sm**, *Summit*; **Ul**, *Ulster*)

APPENDIX C- Bioactivity of traditional and exotic apples varieties harvested in 2006

Table C.1. Antioxidant and antiproliferative activity of apple varieties *

Apples	Cellular antioxidant activity [b]		Antiproliferative activity [c]	
	t-BHP	**H_2O_2**	**HT29**	**MKN45**
Bravo de Esmolfe	47.9 ± 10.1	19.3 ± 2.0	37 .0± 3.7	15 ± 2.0
Malápio Fino	40.2 ± 6.7	19.4 ± 2.5	24.8 ± 2.0	11.9 ± 1.6
Malápio da Serra	36.5 ± 6.7	19.4 ± 2.5	37.0 ± 2.6	19.8 ± 2.0
Pêro Pipo	18.0 ± 4.5	12.6 ± 1.3	49.0 ± 2.0	13.2 ± 1.0
Fuji	29.0 ± 4.4	10.7 ± 1.6	25.8 ± 0.3	17.2 ± 0.9
Gala Galaxy	31.1 ± 4.0	4.1 ± 0.4	48.0 ± 4.3	23.8 ± 1.0
Golden	15.9 ± 1.3	12.6 ± 1.3	42.2 ± 4.6	26.3 ± 2.6
Reineta Parda	23.3 ± 2.6	0.9 ± 0.1	21.1 ± 1.3	9.0 ± 0.6
Starking	18.9 ± 2.3	17.4 ± 2.2	24.1 ± 2.16	15.0 ± 1.1

* Values are expressed as means of at least three replications ± standard deviation;

t-BHP, cellular antioxidant capacity of apple extracts (100 mg/mL) towards t-BHP (2 mM) induced oxidative stress in Caco2 cells; **H_2O_2**, cellular antioxidant capacity of apple extracts (100 mg/mL) towards H_2O_2 (10 mM) induced oxidative stress in Caco2 cells; **HT29**, antiproliferative effect of apple extracts against human colon cancer cells (ED_{50} values expressed as mg/mL); **MKN45**, antiproliferative effect of apple extracts against human gastric cancer cells (ED_{50} values expressed as mg/mL)

APPENDIX D- Characterization of traditional and exotic apple varieties harvested in 2007

Table D.1. Phytochemical composition of traditional and exotic apples harvest in 2007, expressed as mg/100g of edible portion

Compounds	Traditional apples				Exotic apples				
	BE	**MF**	**MS**	**PP**	**F**	**GG**	**G**	**RP**	**S**
Cat	7.70	10.25	1.18	0.57	0.32	1.05	<0.2	0.99	2.30
Ep	12.80	15.44	9.39	10.77	5.38	7.62	4.68	8.98	10.40
CAc	17.3	49.64	26.92	28.02	15.37	15.01	9.43	50.53	11.86
Ph	0.68	0.91	1.82	0.75	0.97	0.53	1.45	11.42	3.71
Q3g	0.096	0.068	0.171	0.164	0.044	0.067	0.107	0.126	0.069
K3g+Q3r	0.296	0.144	0.294	0.416	0.233	0.138	0.381	0.172	0.105
PB1	1.42	1.53	0.80	0.91	0.60	0.82	0.37	0.85	1.15
PB2	2.17	1.60	1.94	5.49	1.73	2.07	2.14	3.17	2.39
TPC	136.5	151.8	118.3	130.1	74.8	82.5	74.5	168.0	139.3

BE, *Bravo de Esmolfe*; **MF**, *Malápio Fino*; **MS**, *Malápio da Serra*; **PP**, *Pêro Pipo*; **F**, *Fuji*; **GG**, *Gala Galaxy*; **G**, *Golden*; **RP**, *Reineta Parda*; **S**, *Starking*; **Cat**, catechin; **Ep**, epicatechin; **Cac**, chlorogenic acid; **Ph**, phloridzin; **Q3g**, quercetin-3-glucoside; **K3g**, kaempferol-3-glucoside; **Q3r**, quercetin-3-rhamnoside; **PB1**, procyanidin B1; **PB2**, procyanidin B2; **TPC**, total polyphenolic content (expressed as mg of gallic acid equivalents)

Table D.2. Antioxidant and antiproliferative activity of apple varieties *

Apples	Cellular antioxidant activity [b]		Antiproliferative activity [c]	
	t-BHP	**H_2O_2**	**HT29**	**MKN45**
Bravo de Esmolfe	48.0 ± 4.0	21.2 ± 3.2	15.5± 0.6	19.3 ± 2.1
Malápio Fino	41.5 ± 1.8	17.3 ± 1.2	21.2 ± 1.9	29.8 ± 2.8
Malápio da Serra	33.8 ± 2.7	20.4 ± 1.1	15.2 ± 2.1	21.6 ± 1.7
Pêro Pipo	19.5 ± 1.3	11.2 ± 1.9	14.1 ± 1.4	13.6 ± 1.2
Fuji	30.1 ± 3.6	13.2 ± 1.9	23.0 ± 2.9	15.0 ± 1.4
Gala Galaxy	35.2 ± 2.3	5.0 ± 1.3	27.8 ± 2.8	28.2 ± 3.4
Golden	18.4 ± 2.1	6.3 ± 0.7	23.5 ± 2.1	24.2 ± 2.8
Reineta Parda	24.6 ± 2.9	1.0 ± 0.2	8.8 ± 0.7	8.6 ± 0.5
Starking	17.6 ± 1.4	17.2 ± 1.3	13.0 ± 2.0	10.1 ± 1.1

* Values are expressed as means of at least three replications ±standard deviation

t-BHP, cellular antioxidant capacity of apple extracts (100 mg/mL) towards t-BHP (2 mM) induced oxidative stress in Caco2 cells; **H_2O_2**, cellular antioxidant capacity of apple extracts (100 mg/mL) towards H_2O_2 (10 mM) induced oxidative stress in Caco2 cells; **HT29**, antiproliferative effect of apple extracts against human colon cancer cells (ED_{50} values expressed as mg/mL); **MKN45**, antiproliferative effect of apple extracts against human gastric cancer cells (ED_{50} values expressed as mg/mL)

In this thesis apples and cherries that are only cultivated in specific regions of Portugal were investigated for their bioactive effects and as sources of functional ingredients with promising application in food, pharmaceutical and nutraceutical industries.